The Role of Interconversion of Scopoletin and Scopolin in Cassava Postharvest Physiological Deterioration (PPD)

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Signed…….. Date……..

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List of Abbreviations

ABRC	: Arabidopsis Biological Resource Center
ACMV	: African cassava mosaic virus
AFLP	: Amplified fragment length polymorphism
APX	: Ascorbate peroxidase
AOX	: Alternative oxidase gene
BGLU	: Beta-glucosidase
BLAST	: Basic local alignment search tool
BSA	: Bovine serum albumin
CaM	: Calmodulin-related calcium sensor protein
CAMBIA	: The Centre for the Application of Molecular Biology to International Agriculture
CaMV	: Cauliflower mosaic virus
CAT	: Catalase
CAZY	: The Carbohydrate-Active enZYme
CBB	: Cassava bacterial blight
cDNA	: complementary deoxyribonucleic acid
CG	: Cyanogenic glycosides
CBM	: Cassava basic medium
CTAB	: Cetyl trimethylammonium bromide
CYSTM	: Cysteine-rich TM
cv	: Cultivar
dbCAN	: Database for automated carbohydrate active enzyme annotation
DIG	: digoxigenin
DMC	: Dry matter content
DNA	: Deoxyribonucleic acid
DW	: Distilled water
EDTA	: Ethylenediaminetetraacetic acid
ESTs	: Expressed sequence tags
FAO	: Food and Agriculture Organization of the United

Nations

FEC : Friable embryogenic callus

FLS : Fibroblast-like synoviocytes

FWD : Forward

F6'H1 : Feruloyl CoA 6' Hydroxylase 1

GD : Gresshoff & Doy

GH : Glycoside hydrolase

GPX : Glutathione peroxidase

HCN : Hydrogen cyanide

HF : High Fidelity

HPLC : High performance liquid chromatography

HPRG : Hydroxyproline-rich glycoproteins

IPTG : Isopropylthio-β-galactoside

iTRAQ : isobaric tags for relative and absolute quantification

LCMS : Liquid chromatography-mass spectroscopy

MAPK : Mitogen-activated protein kinases

MD : Microbial deterioration

Me : *Manihot esculenta*

MS : Murashige & Skoog

MW : Molecular weight

NADPH : Nicotinamide adenine dinucleotide phosphate

NASC : Nottingham Arabidopsis Stock Centre

NCBI : The National Center for Biotechnology Information

NEB : New England Biolabs

OD : Optical density

OPP : Oxidative pentose-phosphate

PAL : Phenylalanine ammonia lyase

PCD : Programmed cell death

PCR : Polymerase chain reaction

PlantCAZyme : Plant Carbohydrate-Active enzyme

PPD : Postharvest physiological deterioration

PS	: Polystyrene
PVPP	: Polyvinylpolypyrrolidone
qPCR	: quantitative polymerase chain reaction
RE	: Restriction enzymes
REV	: Reverse
RNA	: Ribonucleic acid
RNAi	: Ribonucleic acid interference
ROS	: Reactive oxygen species
RT	: Room temperature
RT-PCR	: Reverse transcription polymerase chain reaction
SIGnAL	: Salk Institute Genomic Analysis Laboratory
SBG	: Scopolin-beta-glucosidase
SGT	: Scopoletin-glucosyltransferase
SSA	: Sub-Saharan African
Scopoletin-GT	: Scopoletin-glucosyltransferase
Scopolin-BG	: Scopolin beta-glucosidase
SDS	: Sodium dodecyl sulfate
SLS	: Scientific Laboratory Supplies
SOB	: Super Optimal Broth
SOD	: Superoxide dismutase
SSC	: Sodium Citrate-Saline
TDFs	: Transcript Derived Fragments
T-DNA	: Transferred-Deoxyribonucleic Acid
THT	: Tyramine Hydroxycinnamoyl Transferase
TMV	: Tobacco Mosaic Virus
UGT	: UDP-glycosyltransferase
UV	: Ultra Violet
WB	: Washing Buffer
2OGD	: 2-oxoglutarate-dependent dioxygenase
4-MU	: 4-Methyl Umbelliferone

Abstract

The rapid postharvest deterioration of the roots, known as postharvest physiological deterioration (PPD), has been a major problem to the utilisation and development of cassava (*Manihot esculenta* Crantz) as a food and industrial crop. PPD usually occurs within two to three days after harvest and it is characterised by a blue-black discoloration of the roots, which renders the roots unpalatable and unmarketable. Scopoletin, which is synthesised *de novo* and released from its glucoside, scopolin, during PPD, plays a central role in this discoloration response. Interconversion of scopoletin and scopolin, which is catalysed by scopoletin-glucosyltransferase (scopoletin-GT) and scopolin-beta-glucosidase (scopolin-BG), regulates homeostasis of scopoletin in the cells. However, how this interconversion contributes to root discoloration development is poorly understood. In the present study, we identified and characterised cassava genes for the enzymes that are responsible for the interconversion of scopoletin and scopolin, subsequently manipulated their expression in transgenic cassava through scopoletin-GT RNAi gene silencing and scopolin-BG overexpression constructs. These approaches would potentially alter scopoletin and scopolin content in the root, thereby affecting PPD response. A BLAST search for homologous cassava genes revealed that scopoletin-GT and scopolin-BG are encoded by multiple genes, most of which belong to glucosyltransferase family-1 (GT1) and glycosyl hydrolase family-1 (GH1), respectively. Scopoletin-GT-down-regulated and scopolin-BG overexpressed transgenic cassava lines showed reduced not only scopolin but surprisingly also scopoletin, and delayed PPD. Additionally, other coumarins esculetin and esculin were also identified and both scopoletin-GT, *MeSGT*1, and scopolin-BG, BGLU23, were up-regulated during PPD development at day 4 and day 2, respectively. Our study reveals that disrupting the interconversion of scopoletin and scopolin by inhibiting scopoletin-GT and overexpressing scopolin-BG led to the decrease of both scopoletin and scopolin content and delayed PPD in cassava. These findings provide useful insights into the role of interconversion of scopoletin and scopolin in cassava PPD response and may suggest alternative ways to tackle PPD.

Chapter 1. Introduction and Literature Review

Chapter 1. Introduction and Literature Review

1.1. Introduction

1.1.1. Background of the thesis

Despite the huge potential applications of cassava in many sectors of human life (food, animal feed and industrial products), the roots undergo rapid postharvest deterioration that usually occurs within 2-3 days of harvesting. This deterioration, known as postharvest physiological deterioration (PPD), has become a major constraint to the utilisation and development of cassava roots as a food and industrial crop. PPD is characterised by a blue-black discoloration of the roots that renders them unpalatable for human consumption and unmarketable. In 2016, the world's annual cassava production was projected to be 288.42 million tonnes, with Nigeria becoming the largest producer in the world, followed by Thailand and Indonesia (FAO, 2016). Globally, fresh cassava root losses due to PPD were estimated to be about 5-25% of the total cassava production world-wide (Saravan et al., 2016), although it varies among cassava producing countries (Naziri et al., 2014). In Africa, the estimated physical losses due to PPD was approximately 29%, while in Asia and in Latin America along with the Caribbean about 8% and 10%, respectively (Salcedo and Siritunga, 2011). In Nigeria, delaying PPD for up to two weeks could potentially gain profit of about 2.9 billion USD over a period of 20 years, while in Ghana and Uganda approximately 855 million USD and 280 million USD, respectively (Rudi et al., 2010). Due to these massive physical and economic losses, delaying PPD is one of the most important challenges in cassava production that needs to be addressed in order to meet the increasing demand for cassava.

PPD is a complex endogenous process involving physiological and biochemical reactions that is strongly related to the stress responses to wounding. PPD is initiated by an oxidative burst triggered by the wounding

during harvest, followed by altered gene expression, protein synthesis and production of various secondary metabolites (Buschmann et al., 2000, Huang et al., 2001, Reilly et al., 2003, Reilly et al., 2007, Iyer et al., 2010, Beeching et al., 1998, Owiti et al., 2011). The accumulation of reactive oxygen species (ROS) and hydroxycoumarins, particularly scopoletin, during PPD development plays a major role in the cassava root discoloration (Liu et al., 2017, Uarrota et al., 2015, Sánchez et al., 2013, Xu et al., 2013, Zidenga et al., 2012). Scopoletin forms a blue complex when it is oxidised by peroxidase compounds, such as Hydrogen Peroxide (H_2O_2), which can lead to the root discoloration (Wheatley and Schwabe, 1985, Miller et al., 1975). Thus, PPD is considered as a product of an oxidative process. Therefore, modifying these two components of ROS and scopoletin in cassava could be the key to delay PPD development.

Several efforts have been made to delay PPD progress in cassava via genetic modification. These, so far, have focused on ROS manipulation (Xu et al., 2013, Zidenga et al., 2012). However, a recent study has shown that reducing scopoletin content in cassava roots by modifying a key enzyme, feruloyl CoA 6' hydroxylase, in the biosynthesis of scopoletin could also significantly delay PPD (Liu et al., 2017). Scopoletin is derived from general phenylpropanoid metabolism and some is released from the de-glucosylation of its glucoside, scopolin, in response to stress conditions. In cassava, there are three alternative pathways leading to the biosynthesis of scopoletin (Bayoumi et al., 2008). From the pathway, it shows that the homeostasis of scopoletin is regulated by the interconversion of scopoletin and scopolin, which indicates that this step may also play important role in cassava PPD. However, no research has been reported on how this interconversion might contribute to PPD. Therefore, this thesis focuses on investigating the role of this interconversion in cassava PPD.

The interconversion of scopoletin and scopolin involves two reactions:

glucosylation of scopoletin catalysed by scopoletin-glucosyltransferase (Scopoletin-GT) (Figure 1.1.B) and de-glucosylation of scopolin by scopolin-beta-glucosidase (Scopolin-BG) (Figure 1.1.C).

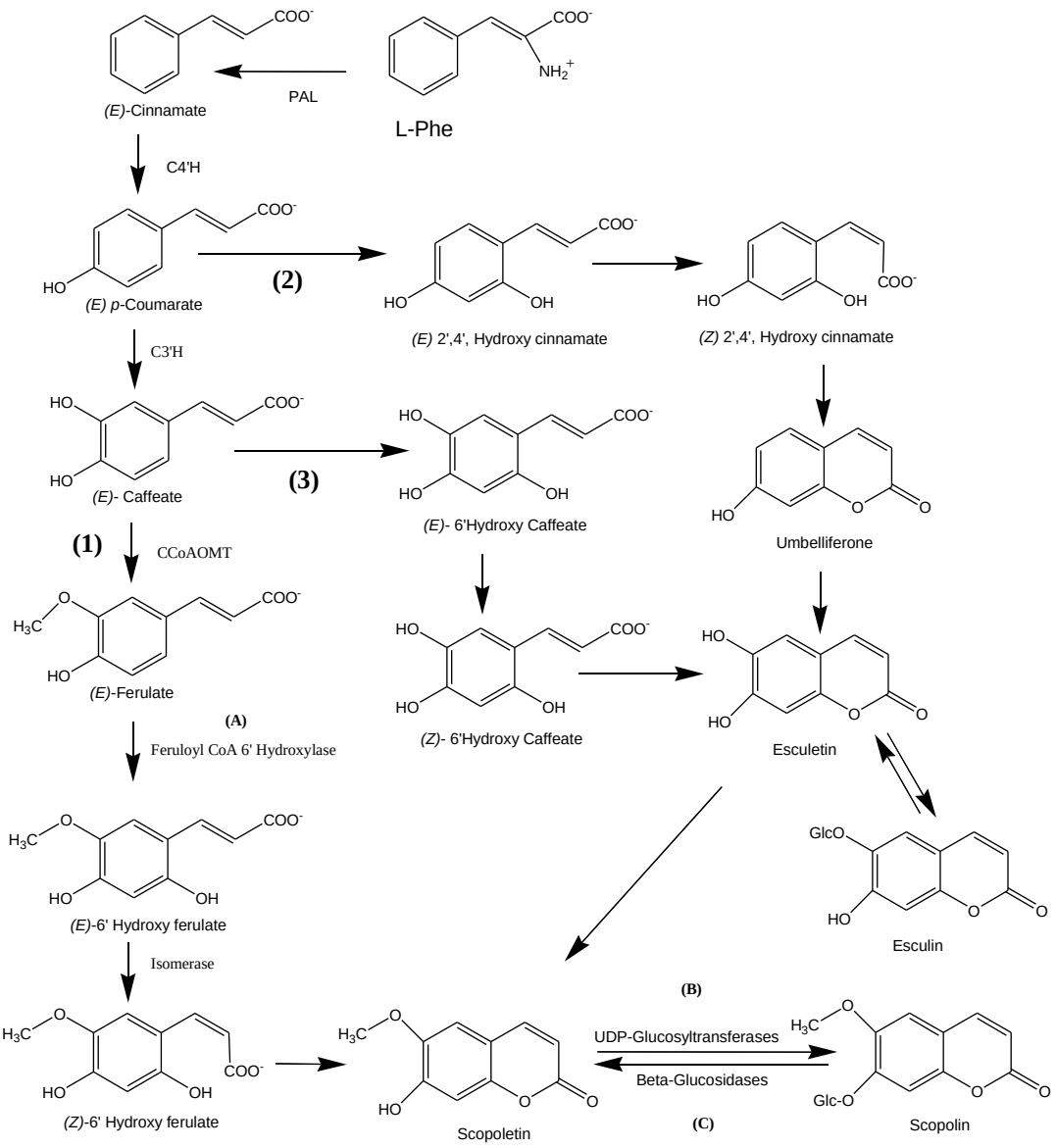


Figure 1.1. Three alternative biosynthetic pathways of scopoletin and scopolin in cassava. The figure was modified from (Bayoumi et al., 2008).

In cassava, the genes for these two enzymes have not been well studied yet. Down-regulation of the gene encoding scopoletin-GT (*togt*1) in tobacco, through the expression of an anti-sense construct, led to the decrease, not

only of scopolin, but also, surprisingly, scopoletin content (Chong et al., 2002). Expression of genes encoding beta-glucosidase from Arabidopsis roots (BGLUs) in insect cells exhibits specific activity towards scopolin and esculin (Ahn et al., 2010). These findings have led us to the identification of homologous gene(s) in the cassava genome and suggested the possibility that manipulation of these cassava gene(s) for both enzymes could also alter scopoletin and scopolin contents in transgenic cassava roots, thereby affecting PPD response.

This study begins with the identification of the genes for scopoletin-GT and scopolin-BG homologous to the reference genes: scopoletin-GT from tobacco, *togt*1, and scopolin-BG from Arabidopsis, BGLU23, in the cassava genome, and followed by the modification of the interconversion of scopoletin and scopolin pathway through scopoletin-GT gene silencing and scopolin-BG gene expression. The results from this study would improve our understanding of the biochemical pathway that affects PPD and may provide alternative solutions to tackle this problem, which will be scientifically and economically beneficial to all stakeholders involved.

1.1.2. Aim of the thesis

This thesis aims to investigate the role of interconversion of scopoletin and scopolin in cassava postharvest physiological deterioration by knocking down cassava scopoletin-GT through RNAi-mediated gene silencing and by overexpressing specific gene for scopolin-BG from Arabidopsis-BGLU23.

1.1.3. Research strategies

To achieve the aim of this thesis, the following strategies were used:

1.1.3.1. Identify and characterise genes encoding scopoletin-GT and scopolin-BG in the cassava genome and select best candidate for genetic modification (Chapter 3).

1.1.3.2. Functional confirmation of the selected cassava genes using

Arabidopsis knock-out mutants (Chapter 4).

1.1.3.3. Transform cassava friable embryogenic calli (FEC) and generate transgenic cassava (Chapter 5).

1.1.3.4. Assess PPD response, analyse gene expression and metabolites changes in scopoletin-GT-RNAi-expressing transgenic cassava lines (Chapter 6).

1.1.3.5. Assess PPD response, analyse gene expression and metabolites changes in scopolin-BG-expressing transgenic cassava lines (Chapter 7).

1.2. Cassava (*Manihot esculenta* Crantz)

1.2.1. Introduction

Cassava (*Manihot esculenta* Crantz) is one of the most important crops in the globe that is widely grown for its storage roots and mainly used for human consumption (Beeching, 2013, Falade and Akingbala, 2011). It provides a staple food for approximately 800 million people worldwide, mostly in Africa and Latin America (Alves, 2002, Liu et al., 2011). Due to its agronomic traits, such as tolerance to drought and good performance in marginal conditions where other crops fail, cassava has been well-known to play a key role in food security in the tropics (Falade and Akingbala, 2011). In addition, cassava is also used for animal feed and as raw material for various industries such as production of starch and biofuel (Liu et al., 2011, Balagopalan, 2002, Sriroth et al., 2010), bread (Pasqualone et al., 2010), snacks (Vitrac et al., 2002), paper, textile, oil-drilling, pharmaceutical and adhesive manufacturing industries (Vlaar et al., 2007). Cassava is the second most important source of starch after maize and no other source of starch is traded more in international markets than cassava starch, which is also called tapioca (Stapleton, 2012). The importance of cassava in many sectors of human life has made it one of the fastest expanding food crops and most attractive crops to be explored by many scientists on this planet (Lebot, 2009, El-Sharkawy, 2012, FAO, 2016). The annual world's cassava production is increasing year by year as an increase in market demand. In

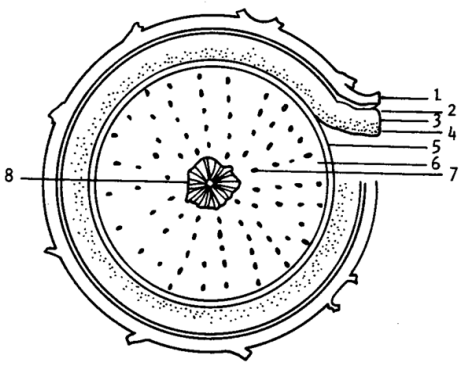
2016, it was projected to be 288.42 million tonnes with Nigeria taking the global lead with a production of about 57.85 million tonnes (FAO, 2016). Despite its benefits, cassava utilisation suffers from two major constraints. Firstly, cassava contains high levels of cyanogenic glycosides (CGs) (Zidenga et al., 2017) that can release toxic cyanide, which is harmful for food consumption if it is not cooked properly. Secondly, the storage roots undergo rapid deterioration within 2-3 days after harvest that renders the roots unpalatable and unmarketable (Beeching et al., 2000).

1.2.2. Taxonomy and morphology

Cassava (*Manihot esculenta* Crantz), a diploid plant with 36 chromosomes, belonging to one of the largest dicot families, Euphorbiaceae (Lebot, 2009, Webster, 1994, Fregene et al., 1997). The family euphorbiaceae consists of about 300 genera and 8000 species, most of which grow in the tropics and produce latex (Lebot, 2009). Classification of the genus *Manihot* has been developed under the *Manihotae* tribe based on multivariate analysis of 44 morphophysiological traits and classifies the 98 species in 19 different sections, which *Manihot esculenta* Crantz is the most cultivated species throughout the tropics (Rogers and Appan, 1973).

Although cassava shows highly variable in the morphological characteristics due to a high degree of interspecific hybridization (Lebot, 2009, Alves, 2002), some common morphological characteristics including leaf, stem and root are described as follows. Cassava leaves are formed by the lamina and petiole and are covered by a shiny and waxy epidermis. The number of leaf lobes is usually uneven, ranging from three to nine. The petiole length of a fully opened leaf varies between 2-30 cm. Cassava produces both male (pistillate) and female (staminate) flowers on the same plant. The mature stem is woody, cylindrical and formed by alternating nodes and internodes. The cassava root is anatomically a true root, not a tuberous root; therefore, it cannot be used for vegetative propagation (Alves, 2002) as potato can.

Figure 1.2 illustrates the structure of cassava root contains several sections or layers such as periderm and schlerenchyma that form the peel, phloem, cambium, parenchyma and xylem vessels.



1. Periderm
2. Schlerenchyma
3. Cortical parenchyma
4. Phloem
5. Cambium
6. Parenchyma
7. Xylem vessels
8. Xylem bundles and fibres

Figure 1.2. Structure of cassava root (Hunt et al., 1977).

The roots are usually harvested within eight to twenty-four months after planting, depending on the variety, condition of growth, nutritional needs and socio-economic context of cultivation (Isendahl, 2011), and the cassava plant can grow up to 4 m high (Figure 1.3).

Due to the large number of cassava cultivars found in farmers' fields, it becomes challenging to describe precisely the morphological variation of each cultivar. Therefore, a tool is needed to help with it, which can be done using morphological and agronomic descriptors. There are various descriptors, among which the following are the basic descriptors that can be used for the characterization of a cultivar: 1) apical leaf colour and pubescence, 2) central lobe shape, 3) petiole colour, 4) stem cortex and external colour, 5) root peduncle presence, 6) root epidermis texture, 7) root flesh, cortex and external colours and 8) flowering (Alves, 2002).

Figure 1.3. Biology of cassava (*Manihot esculenta* Crantz). (a-b) eight month-old field grown cassava plants, Indonesian variety Adira 1, (c) male flower, (d) cassava storage roots, (e) female flower, (f) fruit. Photo credit: Fathoni, A (Beeching, 2013).

1.2.3. The origins and domestication

The origins of cassava (*Manihot esculenta* Crantz) have remained unresolved and debatable until the end of the 20th century (Olsen and Schaal, 1999). According to de Candolle and Vavilov's theories on the geographical origin of cassava, Brazil is most likely to be the area of origin due to the greatest intraspecific variability found in this region (Rogers, 1965). Using a single-copy of nuclear gene glyceraldehyde 3-phosphate dehydrogenase (*G3pdh*), Olsen and Schaal (1999) have examined the

phylogeography of cassava and provided the evidence that the southern Amazon border region as the crop's geographical origin of domestication. In addition, botanical mapping of the current distribution of this crop suggests that the Neotropical lowland savannas to the south of Amazon rain forest, the Cerrado of the Brazilian states of Rondonia and northwestern Mato Grosso is the geographical origin of cassava (Isendahl, 2011).

Cassava might have been domesticated approximately 10,000 years ago, in the early Holocene, or by 7000 BC (Olsen and Schaal, 1999, Isendahl, 2011). Domestication of cassava across the Brazilian states itself was supposed to be done several times before it was distributed around the world (Allem, 2002, Ugent et al., 1986). In the middle of the 16th century, Portuguese traders brought cassava out of the area of origin, Brazil, to Africa. Since then, cassava was widely cultivated across much of the continent, becoming a main food source for people in Africa, particularly in Nigeria (Jones, 1969). During the same century, Portuguese traders also travelled to India and introduced cassava there. In the 17th century, cassava was first introduced to the Philippines by Spanish traders who brought it directly from Mexico and then to Indonesia during the next century. From Jakarta, cassava was transported to Malaysia in the middle of the 18th century. As in Africa, since cassava introduction in the 18th century, its spread was rapid in South-east Asia during the following century (Lebot, 2009).

Today, cassava is grown in all the tropic regions around the world and playing an important role in food security and supplying a raw material for various industries. The agronomic traits of cassava such as its ability to grow in marginal conditions where other crops fail, its vegetative propagation using stem cuttings and a wide range of potential applications, have greatly contributed to its spread and importance as a food crop and also as an industrial commodity (Lebot, 2009, Alves, 2002).

1.2.4. Importance of cassava as a crop

Cassava has become one of the most essential crops in the world due to its huge potential uses. Cassava was firstly introduced to the world as a food crop. Although the leaves, which are rich in calcium and protein, can be utilised as a vegetable, cassava is mainly cultivated for its starchy storage roots (Lebot, 2009). The roots contain high carbohydrate as a source of energy. Compared to other root and tuber crops such as sweet potato, yam and potato, cassava roots are the highest energy source about 160 kcal/100 g as shown in table 1.1 (Chandrasekara and Josheph Kumar, 2016).

As a food crop, cassava plays a key role in food security, particularly in African countries, where cassava is used as the main food source. It provides a staple food for approximately 800 million people worldwide (Liu et al., 2011). In addition, cassava can be used as animal feed and many other purposes. Nowadays, cassava is widely utilised as raw material for various industries such as starch, bioethanol, food-processing, paper, textile, pharmaceutical and adhesive manufacturing industries (Vlaar et al., 2007). It also has potential health benefits due to bioactive constituents such as phenolic compounds that can act as anti-inflammatory, antibacterial or antioxidant (Chandrasekara and Josheph Kumar, 2016). Those have turned cassava to one of the most attractive crops on earth.

Table 1.1. Comparison of several chemical compositions between raw cassava and other tuber and root crops.

Nutrient (per 100 g)	Cassava	Potatoes		Sweet potatoes	Yam
		White flesh and skin	Red flesh and skin		
Energy (kcal)	160	69	70	86	118
Carbohydrate (g)	38.1	15.7	15.9	20.1	27.9
Protein (g)	1.4	1.7	1.9	1.6	1.5
Total lipid (g)	0.3	0.1	0.1	0.1	0.2
Dietary fibre (g)	1.8	2.4	1.7	3.0	1.8
Calcium (mg)	16	9	10	30	17
Magnesium (mg)	21	21	22	25	21
Potassium (mg)	271	407	455	337	816
Vitamin C (mg)	20.6	19.7	8.6	2.4	17.1
Vitamin A (IU)	13	8	7	14187	138
Vitamin K (µg)	1.9	1.6	2.9	1.8	2.3
Vitamin E (mg)	0.19	0.01	0.01	0.26	0.35

Source: (Chandrasekara and Josheph Kumar, 2016)

1.2.5. Production and its challenges

The importance of cassava is also shown by the increase in annual world production of cassava and market demand every year. According to the FAO (2016), the global production of cassava was estimated around 278.7 million tonnes in 2014 with Nigeria was the largest producer in the world approximately 47.4 million tonnes followed by Thailand (30.2 million tonnes), Indonesia (23.9 million tonnes) and Brazil as the origin of cassava in the fourth position (21.5 million tonnes). In 2016, the production was projected to be 288.42 million tonnes and Nigeria is still the largest cassava producing country in the world (Table 1.2).

Table 1.2. Top 10 cassava producing countries.

	2013	2014*	2015**	2016**
		Million tonnes		
World	263.17	278.67	281.05	288.42
The top 10 cassava producing countries				
Nigeria	47.41	54.83	57.00	57.85
Thailand	30.22	30.02	32.35	31.81
Indonesia	23.94	23.44	22.91	26.75
Brazil	21.48	23.24	22.78	22.41
Rep. of Congo	16.50	16.81	15.30	15.20
Angola	16.41	7.64	7.72	7.78
Ghana	15.99	16.52	17.21	17.95
Vietnam	9.76	10.21	10.67	10.20
Cambodia	8.00	7.93	11.94	13.22
India	7.23	8.14	8.00	7.84

* = estimated ** = predicted (FAO, 2016)

Despite its beneficial agronomic traits, the production and utilisation of cassava are limited by several factors such as high amounts of cyanogenic glycosides content, pests and diseases and rapid deterioration of the root after harvest. Cassava has a potential toxicity where both root and leaf contain cyanogenic glycosides (CGs) mostly linamarin that can release hydrogen cyanide (HCN), which is toxic to humans. Therefore, cassava must be cooked or processed properly to get rid of HCN prior to consumption. Cyanogenesis can occur spontaneously or enzymatically, taking place upon tissue disruption, when linamarin which, is stored in the vacuole is transported out of the cells and reacted with an endogenous enzyme, linamarase, to release cyanogens including acetone cyanohydrin, CGs and HCN (Lebot, 2009). This trait is genotype dependent, which means every cassava genotype contains different concentration of total cyanogens ranging from 20 mg/kg to more than 4000 mg/kg dry weight of the roots.

Based on this trait, cassava is classified into two groups, sweet and bitter cassava. Cassava genotypes with total cyanogens less than 100 mg/kg fresh weight are categorised as sweet cassava, whereas those with more than 100 mg/kg fresh weight are bitter cassava (Chiwona-Karltun et al., 2004).

One of the major constraints to cassava production that causes failure in plant growth or harvest is that of pests and diseases. A large number of different pests and diseases in cassava have been identified. The characteristics and impact of those factors vary between the cassava producing countries; South America, and Africa are the continents most affected by the attack of pests and diseases, while in Asia, the effect of pest and diseases on cassava production is less (Lebot, 2009). Cassava is attacked by at least 200 different species of pests, including mites, mealybugs, whiteflies, hornworm, lace bugs, burrower bugs, thrips, scales, fruit flies, shoot flies, gallmidge, white grubs, termites, stem borers, leaf-cutter ants, root mealy bugs, grasshoppers (Bellotti et al., 1999). In addition, nematodes cause damage to parts of plant that leads to bacterial and fungal infections. *Xanthomonas anoxopodis pv. Manihotis* is the pathogen that causes cassava bacterial blight (CBB), a serious disease that can cause a significant impact on cassava production including leaf biomass, storage root production and in planting material, stem (Wydra et al., 2001). There are approximately 250 species of fungi that attack cassava leaves, stems or roots. Among these, *Cercospora henningsii* is the most common one that causes brown leaf spot. This disease is more widespread in all the cassava producing countries, and is characterised by brown spots on both surfaces of the leaf (Lebot, 2009).

Cassava is also vulnerable to a wide range of diseases caused by viruses. At least 17 different viruses have been identified, such as cassava brown streak virus, cassava common mosaic virus, cassava vein mosaic virus,

cassava frogskin virus, and African cassava mosaic virus (Calvert and Thresh, 2002). Amongst them, cassava brown streak virus and cassava mosaic virus are the two most important viruses that cause cassava brown streak disease (CBSD) and cassava mosaic disease (CMD), which have been the most severe and widespread in sub-Saharan African (Ephraim et al., 2015). These two main virus diseases: CBSD and CMD show similar symptom of foliar chlorosis, which makes it difficult to differentiate between them when the plants get infected by both viruses (Hillocks and Jennings, 2003). They have been a major threat to the production of cassava, especially in Africa, where the estimated economic losses due to both CBSD and CMD more than USD 1 billion every year (Legg et al., 2006).

Another major problem to the production and utilisation of cassava is the rapid deterioration of the root shortly after harvest, known as postharvest physiological deterioration (PPD). PPD restricts the storage life of the root to a few days (3-4 days) after harvest and constrains its huge potential uses as a food and industrial commodity. This can also lead to significant physical and economic losses. Delaying cassava PPD would have a significant beneficial impact to the farmers and all stakeholders involved. PPD, which is the main focus of this study, will be reviewed in the following section.

1.3. Postharvest Physiological Deterioration (PPD) in cassava

1.3.1. Introduction

Compared to other root and tuber crops such as sweet potato, yam, and taro, cassava has the shortest storage life after harvest (Lebot, 2009). Once the roots are harvested and detached from the main stem, they undergo the rapid deterioration process of PPD. This phenomenon usually occurs within three days after harvest and renders the roots unpalatable and unmarketable (Beeching et al., 2000). Cassava PPD is firstly triggered by mechanical damage during harvest, and then by postharvest handling, transportation, and storage. PPD is characterised by a blue-black

discoloration of the parenchyma and xylem vessels, known as vascular streaking, initiated from the wounding site and spreading through the parenchyma tissue of the whole root over the storage time (Rickard and Gahan, 1983) as shown in figure 1.4. Neither bacteria nor fungi are isolatable from freshly harvested cassava roots and treatments using bactericide and fungicide were not able to stop the deterioration process, implying that PPD is an endogenous physiological rather than microbial or pathological response (Noon and Booth, 1977, Venturini et al., 2015).

A **B**

Figure 1.4. The visual symptom of cassava PPD. Root discoloration of the root between A) fresh root (day 0) and B) deteriorating root (day 4).

PPD is typically followed by the second type of deterioration caused by the invasion of various microorganisms or pathogens, known as microbial deterioration (MD), which occurs after 5-7 days of harvest (Booth, 1976) as shown in figure 1.5.

Figure 1.5. Cassava postharvest deterioration caused by microorganisms, known as microbial deterioration (MD).

PPD is a complex process due to many reactions involved during its progress and to various factors affecting its nature and development. PPD is strongly correlated with the wound stress response induced by mechanical injury or physical root damage during harvest. This trait is influenced by environmental factors, such as growth conditions and storage of harvested roots (Zidenga et al., 2012, Tumuhimbise et al., 2014). These make PPD more complex and difficult to fully understand its mechanism, and to tackle the problem by inhibiting a single factor. Much effort to understand the mechanisms of PPD and to delay it using conventional as well as molecular approaches has occurred over the last few decades. However, this problem is still not fully solved. PPD has been an important problem because it has a significant impact, physically and economically, to farmers and all stakeholders (Beeching et al., 2000).

1.3.2. Physiological and biochemical responses during PPD

PPD is an endogenous physiological and biochemical process, essentially linked to wound response, comparable to those observed in other plants (Rickard and Gahan, 1983). During PPD, various reactions take place including rapid production of ionic oxygen (reactive oxygen species, ROS), changes in gene expression and protein synthesis, activation and de-activation enzymes related to wound responses, and accumulation of secondary metabolites, some of which have anti-microbial or anti-oxidant activity (Qin et al., 2017, Uarrota et al., 2016, Hu et al., 2016, Vanderschuren et al., 2014, Owiti et al., 2011, Reilly et al., 2007, Reilly et al., 2003, Buschmann et al., 2000). PPD is also accompanied by an increase in respiration, an increase in sugar content due to starch degradation, changes in lipid composition, and cell wall repair (Han et al., 2001). PPD is induced by mechanical injury or physical root damage during harvest (Beeching et al., 1994). The initial injury stimulates the cells to release signal molecules leading to the production of various compounds that play roles in different pathways including tissue cell protection, defense as well as restoration.

The signal molecules such as ethylene, jasmonic acid, abscisic acid, salicylic acid, hydrogen peroxide (H_2O_2) are either synthesised *de novo* or as a product of inactive precursor hydrolysis induced by the wounding (Beeching et al., 1994, Han et al., 2001).

1.3.2.1. Oxidative burst and PPD

PPD is the product of an oxidative process (Reilly et al., 2003). Its visual symptom, blue-black discoloration of the xylem vessels and parenchyma of cassava roots, is due to the oxidation of phenolic compounds, mostly scopoletin, that are rapidly produced after injury (Buschmann et al., 2000, Wheatley and Schwabe, 1985). Reactive oxygen species (ROS) production occurs during cassava PPD in response to wounding and play a role in PPD development. ROS are normally continuously generated as by-products of photosynthesis and play roles in various processes in plant metabolic pathways such as growth, the cell cycle, programmed cell death, hormone signalling, biotic and abiotic stress responses. However, they are also capable of causing oxidative damage to the cells (Mittler et al., 2004, Bhattacharjee, 2005). Under normal conditions, plants have several mechanisms to scavenge ROS, preventing their potential toxicity. However, Disruption of the equilibrium between production and scavenging of ROS is resulting in a rapid production of reactive oxygen species (ROS), known as the oxidative burst (Apostol et al., 1989).

An oxidative burst is one of the earliest events leading to cassava PPD development (Iyer et al., 2010). Superoxide (O_2^-) is the first free radical form detected within the first 15 min upon tissue disruption or injury, followed by hydrogen peroxide (H_2O_2) within 3 h after injury (Reilly et al., 2003). Interaction between this peroxide (H_2O_2) and secondary metabolites mainly scopoletin, resulting in a blue-black pigmentation observed as PPD. Delaying ROS production by enhancing gene expression of the ROS-scavenging enzymes catalase (CAT) with superoxide dismutase (SOD)

delayed PPD development (Xu et al., 2013). SOD and CAT work together to remove ROS, where SOD converts superoxide (O_2^-) to hydrogen peroxide (H_2O_2) and then CAT converts the latter ROS to water and oxygen. Increases in ascorbate peroxidase (APX) and glutathione peroxidase (GPX) occur during PPD (Uarrota et al., 2016). The latter two enzymes are also able to convert hydrogen peroxide to water molecule (Apel and Hirt, 2004) (Figure 1.6).

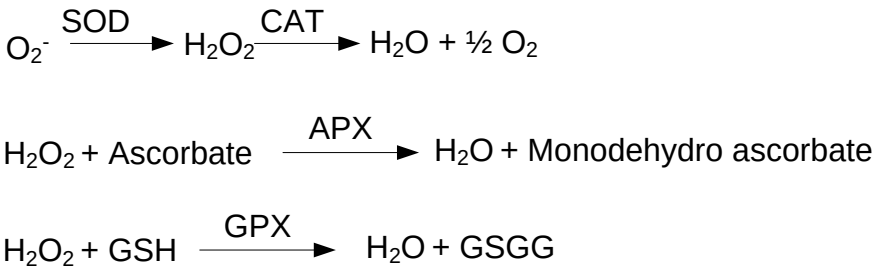


Figure 1.6. The principle mode of enzymatic ROS scavenging by superoxide dismutase and catalase (Apel and Hirt, 2004).

The oxidative burst contributes to the response and status of the disrupted tissues or cells because ROS can act in various ways. For instance, ROS are generated as signalling compounds to manage various events due to stress conditions such as production of defensive or restorative compounds, and programmed cell death. Interaction between ROS and the cells could lead the oxidative damage to protein, DNA and lipids (Apel and Hirt, 2004). Therefore, another strategy to delay PPD is by preventing the excessive production of ROS in the cells. Cyanogenesis is highly associated with the oxidative burst that triggers PPD (Zidenga et al., 2012). Upon disruption, the tissues undergo cyanogenesis releasing hydrogen cyanide (HCN) from linamarin and linamarase. HCN inhibits complex IV in the mitochondrial electron transfer chain and blocks mitochondrial respiration. This inhibition causes a rapid and massive production of ROS at complex I and III. Therefore, PPD could be cyanide-dependent. Overexpression of

mitochondrial alternative oxidase gene (*AOX1A*) from *Arabidopsis thaliana* in cassava reduced ROS accumulation 10-fold and delayed PPD (Zidenga et al., 2012). In addition, recent findings showed that homeostasis of cellular ROS in the cells can be controlled by melatonin. Applying exogenous melatonin significantly delayed physiological deterioration in the cassava root (Ma et al., 2016, Hu et al., 2016). Melatonin acts through activation of ROS-scavenging and ROS signal transduction pathways including antioxidant enzyme, calcium signalling, mitogen-activated protein kinases (MAPK) cascades and transcription factors at early stage of PPD. It plays role in reducing H_2O_2 by improving catalase and peroxidase activity, repressing starch degradation at middle and late stages. Therefore, manipulation of melatonin biosynthesis pathway could potentially delay PPD (Hu et al., 2016).

1.3.2.2. Gene expression and protein profiles during PPD

PPD is an active process involving up- and down-regulation of many genes. These genes are involved in various cellular processes, some of which may play a direct role in PPD development such as signal transduction pathway, reactive oxygen species (ROS) turn over, and phenylpropanoid metabolism that leads to the production of phenolic compounds such as scopoletin that play a major role in blue-black discoloration, PPD. They are also involved in other cellular processes in response to wounding including cell wall repair, programmed cell death (PCD), carbohydrate and lipid metabolism and ion, water, or metabolite transport (Qin et al., 2017, Vanderschuren et al., 2014, Owiti et al., 2011, Reilly et al., 2007). This successful identification of the genes expressed or transcript levels during PPD has improved significantly our understanding of the molecular basis of PPD.

However, these extensive insights into changes in gene expression are not sufficient to fully understand PPD mechanisms. Very early studies of PPD have revealed that this phenomenon is associated with changes in several

enzymes activities such as phenylalanine ammonia lyase (PAL), peroxidase, catalase (CAT), and superoxidase dismutase (SOD) (Rickard and Gahan, 1983, Tanaka et al., 1983). Application of the protein-synthesis inhibitor, cyclohexamide, on freshly harvested root inhibited PPD development (Uritani et al., 1984a). This suggested that PPD is an active process involving protein synthesis. Recent studies show that PPD is a highly regulated complex process involving various proteins (Owiti et al., 2011, Vanderschuren et al., 2014). In addition, because transcript accumulation does not always correlate with protein accumulation, it is important to investigate further changes in protein accumulation, interactions, modifications and activities during PPD development (Vanderschuren et al., 2014). With the use of isobaric tags for relative and absolute quantification (iTRAQ) technique, Owiti et al (2011) identified a massive number of proteins that were either up-regulated or down-regulated in early and later PPD. In the early PPD response, 67 proteins were up-regulated while 141 were down-regulated. While in the later stage, 179 proteins were up-regulated and 170 were down-regulated. Amongst these, 48 proteins and 20 proteins were up-regulated during both stages. The up-regulated proteins during PPD play roles in ROS scavenging pathways, including PCD pathway, defence pathway, cell wall metabolism and signalling pathway (Figure 1.7). Those also include novel proteins such as linamarase, glutamic acid-rich protein, hydroxycinnamoyl transferase, glycine-rich RNA binding protein, β-1,3-glucanase, pectin methylesterase, maturase K, dehydroascorbate reductase, allene oxide cyclase, and proteins involved in signal pathways (Owiti et al., 2011).

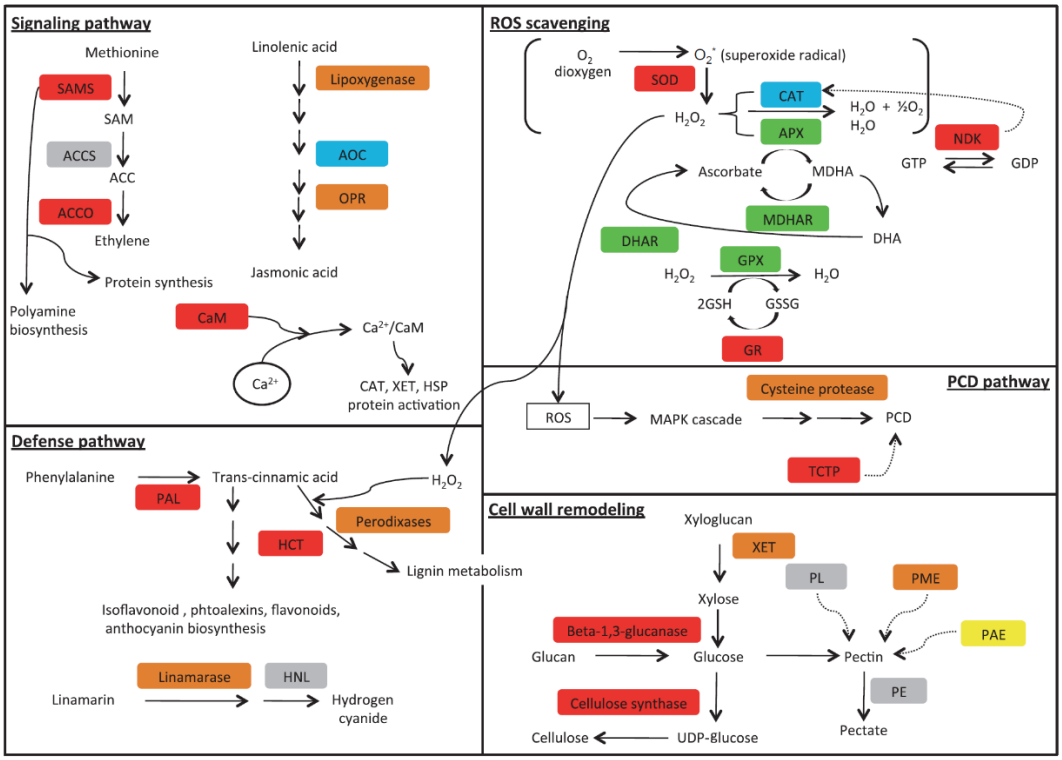


Figure 1.7. Model of the biological pathways affected during cassava root deterioration (PPD) (Owiti et al., 2011).

Additionally, Vanderschuren et al (2014) identified over 2600 unique proteins in the cassava root, with more proteins (approximately 300 proteins) identified than previous studies and showing abundance regulation during cassava PPD development. These were found to be associated with oxidative stress, phenylpropanoid biosynthesis (including scopoletin), the glutathione cycle, fatty acid a-oxidation, folate transformation, and the sulphate reduction II pathway, glutathione-associated enzymes, including glutathione reductases, glutaredoxins, and glutathione S-transferases (Vanderschuren et al., 2014). They identified glutathione peroxidase (GPX) as a candidate for genetic modification to delay PPD. Overexpressing a cytosolic glutathione peroxidase in cassava reduced lipid peroxidation as well as decreased H_2O_2 accumulation, and delayed PPD (Vanderschuren et al., 2014). While these studies have

provided valuable insights through analysing changes in transcript or protein populations, they have not focused on specifically of particular pathways known to be important in PPD, such as parts of general phenylpropanoid metabolism.

1.3.2.3. Secondary metabolites produced during PPD

During PPD, a range of secondary metabolites accumulates that includes some with protective or defensive functions. The visual symptom of PPD, blue-black discoloration, is accompanied by a rapid accumulation of fluorescent compounds in the parenchyma of the cassava root (Salcedo and Siritunga, 2011) that can be easily observed under UV light (Figure 1.8).

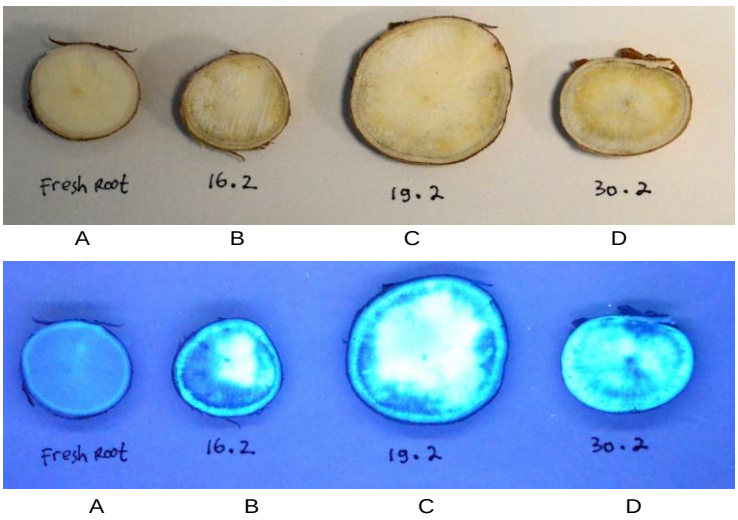


Figure 1.8. Accumulation of fluorescent compounds in the fresh cassava root and deteriorating roots observed by UV light.

Those have been identified as hydroxycoumarins, including scopoletin (6-methoxy-7-hydroxy-coumarin), scopolin (6-methoxy-7-hydroxy-coumaroyl-7-β-D-glucoside), esculetin (6,7-dihydroxycoumarin), and esculin (6, 7-dihydroxycoumaroyl-6-β-D-glucoside) (Tanaka et al., 1983, Uritani et al., 1984b, Buschmann et al., 2000, Bayoumi et al., 2010). Amongst them, scopoletin plays the most important role in cassava PPD development. Peroxidase-mediated oxidation of this compound forms a blue-black

complex (Miller et al., 1975), which is most likely to contribute to root discoloration observed as PPD. A significant increase in the accumulation of scopoletin shortly after injury, more intense fluorescent compound and rapid discoloration in the cassava root were observed when exogenous scopoletin applied (Wheatley and Schwabe, 1985), clearly demonstrating the importance of scopoletin in the wound and particularly in the PPD response. Vanderschuren et al (2014) suggested that the genes for phenylpropanoid pathway including scopoletin biosynthesis could be good candidates to delay PPD. Recently, Liu et al (2017) inhibited a key enzyme, feruloyl CoA 6'-hydroxylase, in the scopoletin biosynthesis pathway via RNAi gene silencing, that confirmed the importance of the coumarin, scopoletin in PPD. As scopoletin is also released from its glucoside, scopolin via de-glucosylation by the action of beta-glucosidase, it is equally important to study the interconversion of scopoletin and scopolin, which becomes the main topic of this thesis.

In addition to coumarins, some other metabolites were also produced in association with PPD and in response to wounding. In the previous section, we have shown that PPD involves up-regulation of many genes and proteins or enzymes, one of which is phenylalanine ammonia lyase (PAL). This enzyme is the first enzyme in the phenylpropanoid metabolism that leads to the production of various important secondary metabolites in response to wounding or during PPD including scopoletin, scopolin, esculetin, esculin, flavan-3-ols, (+) catechin, (+) gallocatechin, lignin-like, lignin, suberin, flavonoids, anthocyanins and terpenoids (Beeching et al., 2000, Tanaka et al., 1983, Wheatley et al., 1985, Beeching et al., 1999, Uarrota et al., 2014).

1.3.2.4. Other events during PPD

PPD, which involves a wide range of responses, has shown commonality with wound response in other plants (Beeching et al., 1999, Rickard, 1985). Many other events which may or may not have direct effect on PPD development were also observed such as cyanogenesis, lipid degradation, starch degradation, programmed cell death (PCD), and wound repair.

Cyanogenesis

Cyanogenesis has clearly shown to play a key role in inducing oxidative burst by blocking the mitochondrial respiration which has been reviewed in section 1.3.2.1 (Zidenga et al., 2012).

Lipid degradation

In the damaged tissue, the membrane lipids presumably have been broken down, which can be observed from the changes in total phospholipids and glyceroglycolipids as well as sterol-containing lipids during PPD development in cassava (Beeching et al., 1999). The interaction between membrane lipids in damaged tissue with ROS such as H_2O_2 can lead to the production of long chain fatty acids including linolenic that acts as a precursor in the biosynthesis of jasmonic acid, a signalling molecule in wound response in other plants (Farmer and Ryan, 1992).

Starch degradation and sugar accumulation

A consistent increase in total soluble sugars, such as glucose and fructose, during PPD progress (Uarrota et al., 2015, Sánchez et al., 2013) shows that starch degradation is a part of the wound response. Higher sugar content was found to be positively correlated with the delayed PPD in cassava (Sánchez et al., 2013).The relationship between higher sugar levels and delayed PPD was also shown in pre-harvest pruning of cassava roots (van Oirschot et al., 2000). Soluble sugars are known to be involved in response to various stresses and act as nutrient and metabolite signalling molecules

that activate specific or hormone-crosstalk transduction pathways resulting in changes of gene expression and protein synthesis (Couée et al., 2006). They can play a dual role by involving in, or related to, ROS-producing metabolic pathways, and, conversely, in NADPH-producing metabolic pathways such as the oxidative pentose-phosphate (OPP) pathway, which contributes to ROS scavenging (Couée et al., 2006). In mammalian cells, high glucose induces production of ROS and mitochondrial swelling that can lead to neural apoptosis through the activation of caspase-3 and -9 (Russell et al., 2002). While low glucose led to the decrease of mitochondrial ROS production and the increase of chronological life span in yeast (Barros et al., 2004). Contrary, glucose enhances cellular defences against cytotoxicity of hydrogen peroxide (H_2O_2) in certain mammalian cell types (Averillbates and Przybytkowski, 1994). High glucose can lead to the activation of NADPH oxidase in the OPP pathway, which is a major cofactor of ROS scavenging pathways such as ascorbate-glutathione cycles. The level of glutathione has shown to correlate with the plants' adaptation to various stress conditions (May et al., 1998). Moreover, different types of sugar such as glucose and sucrose may or may not have the same effects in oxidative stress responses. For example, sucrose feeding increases ascorbate levels in harvested broccoli florets while glucose does not (Nishikawa et al., 2004). By the action of ascorbate peroxidase (APX) enzyme, ascorbate converts H_2O_2 to water. Although sugars seem to play important roles in wound responses or delayed PPD, the precise role of sugars in cassava PPD response has been little studied.

Programmed cell death (PCD)

Programmed cell death (PCD), also known as apoptosis is an active process that regulates cellular suicide processes. Unlike necrosis, cell death caused by extrinsic factors, PCD process involves new protein synthesis which may play essential roles in growth, development and survival in eukaryotes (Djabou et al., 2017, Van Breusegem and Dat, 2006). Moreover,

PCD is responsible for cell death in response to pathogens and various abiotic stresses (Hoeberichts and Woltering, 2003). The common biochemical and morphological symptom when the cells undergo PCD are compaction and shrinkage of the cytoplasm and nucleus, DNA and nuclear fragmentation into 50-300 kb long, and calcium influx (De Jong et al., 2000, Wang et al., 1996).

PCD process is likely to be activated during PPD development via the up-regulation of cysteine protease that could enhance protease activity leading to PCD (Owiti et al., 2011). Iyer et al (2010) also identified the genes for enzymes involved in programmed cell death expressed within 4 h after harvest. In addition, the up-regulation of calcium sensors proteins, CaM, at the early stage of PPD (Owiti et al., 2011) could potentially lead to a rapid increase in Ca^{2+} levels in the cells resulting in the oxidative burst, as occurs in *Arabidopsis thaliana* (Kaplan et al., 2006). The elevated cytosolic Ca^{2+} levels could induce NADPH oxidase activity, which plays a key role in oxidative burst and PCD process. ROS play an important role as signalling compounds in the activation of plant PCD (Hoeberichts and Woltering, 2003). ROS can be harmful to organisms when it is at high concentrations causing cells damage resulting in loss of physiological capacity and subsequently cell death (Djabou et al., 2017).

In addition to ROS, accumulation of scopoletin during PPD may also lead to apoptosis or PCD processes, although the relationship between scopoletin and PCD during PPD has not been explored. Scopoletin does induce apoptosis of fibroblast-like synoviocytes (FLS) from adjuvant arthritis rats (Li et al., 2009) and human promyeloleukemic cells (Kim et al., 2005). Li et al (2009) have found that scopoletin induces apoptosis of rat FLS via a mitochondrial-dependent pathway, mediated by the activation of caspase-3 and reduction of the activation of NF-kB proteins. A proteolytic cascade appears to be the key component of the apoptotic machinery, which

involves a family of cysteine proteases, namely **c**ysteine-**asp**artic acid prote**ase** or caspases (Hoeberichts and Woltering, 2003). In cassava, the up-regulation of cysteine protease (MecCP1, AY973616) during PPD (Reilly et al., 2007, Owiti et al., 2011) may represent the expression of caspase-3 or caspase-3-like proteins, although the specific caspase-3 genes from cassava have not been definitively characterised. Cysteine-rich TM module stress tolerance (CYSTM) has been recently identified in cassava (Qin et al., 2017). More studies are required on the identification of caspase-3 genes in cassava and the effect of exogenous scopoletin on their expression might help us understand more the role of scopoletin in PCD process during PPD development in cassava.

Wound repair

Cassava PPD resembles plant wound stress responses found in other plant species (Rickard, 1985, Beeching et al., 2000). In addition to the production of signalling compounds and defensive compounds that have been reviewed in the earlier sections, cassava roots also produce barrier layers or reparative compounds such as suberin and lignin to heal the damaged tissue and also to protect from potential microorganism invasion (Beeching et al., 1999). Wound repair, also known as curing, occurs in cassava upon tissue disruption, such as the up-regulation of hydroxyproline-rich glycoproteins (HPRGs) during PPD and the activation of PAL enzymes shortly after injury that leads to the production of various compounds including lignin and suberin (Reilly et al., 2007, Owiti et al., 2011, Han et al., 2001). In fact, cassava roots are able to repair the wounded tissue when the storage roots are still attached to the main stem. However, this ability to repair becomes inadequate once the roots are detached from the main stem (Beeching et al., 2000). The sealing and healing of wound sites does occur in the detached harvested roots, but the process appears be too slow under normal conditions of storage. However, under optimum storage conditions of 30-35 °C and 80-85% relative humidity (RH) allows curing to occur in the

wounded harvested cassava roots (Wheatley, 1989, Beeching et al., 1994). Failure in repairing the damaged tissue may lead to the following responses: an increase of stress-related signalling compounds, rapid production of ROS or oxidative burst, production of defensive compounds including scopoletin that play a major role in the cassava root deterioration (PPD), and microbial access to the root. Thus, cassava PPD is strongly correlated with an inadequate healing process through poor early suberization or lignification (Beeching et al., 1999).

1.3.3. Economic impact of PPD

PPD is a major constraint to the production and utilisation of cassava as a food and industrial crop, causing huge physical and economic losses to farmers and all stakeholders (Vlaar et al., 2007, Beeching et al., 2000). PPD reduces the acceptability of the roots in the market due to decreased starch content and quality, which are crucial for industry and also affects the taste after cooking, which is important for human consumption. These factors lead to the price discount of fresh roots (Naziri et al., 2014); additionally, the roots are becoming totally unacceptable and unmarketable when they have undergone microbial deterioration. In a recent study, the losses due to PPD were projected to be in the range of 5-25% of the total cassava production world-wide (Saravan et al., 2016) although it depends on the countries (Naziri et al., 2014). In Africa, it was estimated the physical losses due to PPD are up to 29%, while in Asia and in Latin America along with the Caribbean about 8% and 10%, respectively (Salcedo and Siritunga, 2011). In Sub-Saharan African (SSA) countries such as Nigeria, Ghana, and Uganda suffer higher PPD losses than in Asian countries because most of the roots are still in the fresh form when they reach buyers or consumers. In Nigeria, delaying PPD for up to two weeks could potentially gain profit of about 2.9 billion USD over a period of 20 years, while in Ghana and Uganda approximately 855 million USD and 280 million USD, respectively (Rudi et al., 2010). In the Northeast of Thailand alone, it was estimated the benefits

in delaying PPD up to 45 days about 26.5 million USD per year for the starch industry and 8.5 million USD per year for farmers (Vlaar et al., 2007).

1.3.4. Factors affecting PPD

Since PPD resembles wound stress responses, physical injury or damage becomes the most important factors initiating the PPD process. The first symptom of PPD is initiated from the wounded site and spreading through the whole root with time (Rickard and Gahan, 1983). This sort of damage is inevitable and usually takes place during harvest and postharvest handling operations (Rickard, 1985, Booth, 1976) including transporting the harvested roots to the end users and storage techniques. Other factors that can influence physical damage of the cassava roots when harvesting in the field are soil compaction, harvesting method (manual or mechanical), root shape, root length, the presence of peduncle (proximal part that is close to the main stem), and peel adherence and texture (Booth, 1975, Diamante, 1986, Salcedo and Siritunga, 2011, Wheatley et al., 1985).

There is a high variability in PPD response between different cassava genotypes and even within the same genotypes, strongly suggested that PPD is genotype dependent but also influenced by environmental factors. Some cassava traits have also been shown to correlate with PPD response, such as carotenoid content, dry matter content (DMC), ratio of sugar/starch, and polyploidy (Uarrota et al., 2015, Uarrota et al., 2014, Sánchez et al., 2013, Moyib et al., 2015). Total carotenoid content in the roots is negatively correlated with PPD (Morante et al., 2010, Sanchez et al., 2006, Rodriguez, 2001). This might be due to the antioxidant properties of carotenes that supress or reduce the oxidative burst (Salcedo and Siritunga, 2011). Negative correlation was also observed between sugar/starch ratio and PPD (van Oirschot et al., 2000), which demonstrates the role of sugar in stress response as reviewed in the section 1.3.2.4. DMC, on the other hand, shows a positive correlation with PPD (Rodriguez, 2001, van Oirschot et al.,

2000, Morante et al., 2010). Recent findings by Moyib et al (2015) found that polyploidy or the presence of multiple genomes within a single nucleus also affects PPD response. Polyploid genotypes showed higher PPD tolerance than diploid genotypes. The presence of extra genome copies in polyploid genotypes might lead to the changes of physiological and increases of genetic buffering that cause unique characteristics (Hegarty and Hiscock, 2007). Not only tolerance to PPD, polyploid genotypes also have shown several better other traits than diploid such as higher tolerance to nutrient stress, and enhanced resistance to environmental stress (Fawcett et al., 2009). Although polyploids can be distinguished by their leaf morphology, which are normally broader, thicker and of a darker green (Lebot, 2009), not all morphoanatomical characters correlate with polyploidy and this trait is species or genotypes dependent (Caruso, 2010).

Furthermore, the PPD response is not only under genetic control but is also influenced by environmental factors, including where the plants are grown (Salcedo and Siritunga, 2011). Evaluation of twelve cassava genotypes in Uganda at three different planting sites and different harvest times have revealed that genotype, location, harvest time, genotype x harvest time, and harvest time x location are highly significantly different for the PPD response (Tumuhimbise et al., 2014). A highly significant interaction between location, season and PPD was also observed by Kawano and Rojanaridpiched (1983). Thus, selection of cassava PPD tolerance is location dependent. Moreover, the development of PPD is also depending on storage techniques and conditions (Sanchez et al., 2006), which will be reviewed in the following section.

1.3.5. Strategies to tackle PPD

Those factors that affect the nature and development of PPD have illustrated the complexity of cassava PPD and made it difficult to be completely solved. Many efforts have been tried to prevent and delay PPD,

which can be categorised into three strategies: pre-harvest, breeding, and post-harvest. However, some of them are often unfeasible for economic, and practical (Beeching et al., 1998).

1.3.4.1. Pre-harvest

PPD begins when the cassava roots are harvested and detached from the main stem. Therefore, losses due to PPD can be prevented by harvesting the roots as needed. However, the land would become less productive as land is occupied to maintain the plants and this would not be suitable for large scale farmers. Another strategy is applying a pre-harvest pruning method. This is sort of traditional practice before harvesting which involves removal of all parts of plant including stem and leaves approximately 40-50 cm above the soil, which is usually done 2-3 weeks prior to harvesting the cassava roots (Salcedo and Siritunga, 2011). The pre-harvest pruning has proven to effectively delay cassava PPD. However, this technique has a negative effect on root quality, such as dry matter content, starch quality and quantity (van Oirschot et al., 2000, Tanaka et al., 1984, Hirose et al., 1984, Data et al., 1984). Thus, strategies in this section are only pertinent for small holder farmers.

1.3.4.2. Breeding Programmes

This includes conventional breeding and molecular breeding programmes including the use of molecular markers-assisted selection (MAS) and transgenic approaches. Conventional breeding has been successfully applied in cassava to improve yield or African cassava mosaic virus (ACMV) resistance, but not yet for PPD resistance. The high degree of heterozygosity, few flowers, low pollen fertility, self-incompatibility, and low fruit rate (Ceballos et al., 2010) have made conventional breeding complicated. Moreover, the positive correlation between DMC and PPD makes this method even more challenging.

On the other hand, molecular breeding including molecular markers-assisted selection (MAS) and genetic modification offer better alternative strategies to conventional breeding (Reilly et al., 2003, Taylor et al., 2004, Carmo et al., 2015). Marker-assisted selection (MAS) is used to identify resistance source in the absence of pathogen. This technique has been successfully applied to identify cassava mosaic disease (CMD)-tolerant accessions amongst one thousand two hundred and seventy-four accessions derived from several ecosystems in Brazil, Colombia, Venezuela and Nigeria during the early stages of the breeding program. The use of MAS shortens the detection period of CMD-resistant genotypes to only two years (from crossing to obtaining the F_1 plants) compared to eight years based on conventional breeding (Carmo et al., 2015). In addition to MAS, genetic modification or transgenic approach has proven to be a powerful tool to introduce a number of desired genes for important agronomic traits such as pest and disease resistance, high yield and quality (Liu et al., 2011). Recently, genetic modification has been applied to improve cassava for delayed PPD. In association with PPD, the basic concept of genetic modification might be based on this question: how to stop damaging factors (oxidative burst and scopoletin) and stimulate repair? We have shown that PPD is a product of oxidative process between ROS and coumarins, mainly scopoletin. Therefore, limiting their accumulation in the cassava roots could potentially delay PPD development. Preventing the oxidative burst by reducing ROS production or enhancing ROS-scavenging enzymes activity effectively delay PPD (Xu et al., 2013, Zidenga et al., 2012). Liu et al (2017) have demonstrated that reducing scopoletin content in the cassava roots by inhibiting a key enzyme, feruloyl CoA 6′-hydroxylase, via RNAi silencing could significantly reduce PPD.

These findings have helped us understand better the importance of ROS and scopoletin in cassava PPD. However, RNAi silencing of feruloyl CoA 6′-hydroxylase did not completely block scopoletin production in the root.

Bayoumi et al (2010) identified three possible pathways leading to scopoletin biosynthesis, which is also released from its glucoside, scopolin. Therefore, it is important to investigate this step during cassava PPD process. Additionally, this might be also of interest to combine the two approaches by inhibiting ROS production/enhancing ROS-scavenging and inhibiting scopoletin biosynthesis to produce PPD tolerant cassava lines.

1.3.4.3. Post-harvest

Since PPD is an active process involving oxidation reactions, storing harvested cassava roots under conditions that can prevent the process initiating is crucial. Several successful post-harvest treatments that have been applied including storing the roots at 5 °C or 35 °C with high relative humidity of 80-85%, and dipping the roots in hot water (60 °C) for 45 min (Averre, 1967). As cassava has become more of an industrial commodity, advanced techniques such as the use of polyethylene bags, waxing and deep freezing are being widely and commercially applied (Salcedo and Siritunga, 2011). In some places, we can also find the peeled cassava roots stored in vacuum bags. Several chemical treatments including ethanol (greater than 20%), 10% sodium sulphite, 10% sodium dithiocarbamate, and saturated sodium chloride can delay the discoloration (Noon and Booth, 1977). Recently, treatment with melatonin could also effectively delay PPD progress (Ma et al., 2016, Hu et al., 2016). Whereas, applying bactericide and fungicide only prevent deterioration caused by microorganism (microbial deterioration) (Noon and Booth, 1977). However, while these approaches may be appropriate for consumers prepared to pay a premium for their traditional food, such as ex-patriate or urban middle-class consumers, they are generally uneconomical for wide-scale application for such a low value commodity as cassava.

1.4. Phenylpropanoid metabolism in higher plants

Phenylpropanoid metabolism is one of the most important pathways in plants that leads to the biosynthesis of a wide range of plant natural products including flavonoids, hydroxycinnamic acids, coumarins, stilbenes, lignin and condensed tannins based on the few intermediates of the shikimate pathway as the core unit (Fraser and Chapple, 2011, Dixon et al., 2002, Vogt, 2010). The products of phenylpropanoid pathway have diverse biological functions, such as signal molecules, phytoalexins, structural components, flower pigments or UV protectants, and also function as complex, polymeric constituents of surface and support structures, such as suberin, lignin, and other cell-wall components (Dixon et al., 2002, Vogt, 2010, Hahlbrock and Scheel, 1989). They are also well-known to play vital roles in plant responses to biotic and abiotic stresses (Vogt, 2010); for example, as indicators of plant stress responses upon variation of light or mineral treatment, also key mediators of the plants resistance towards pests (La Camera et al., 2004). These compounds are referred to as secondary metabolites because they are not involved in basal cellular processes such as photosynthesis, respiration, protein and nucleic acid synthesis (Fraser and Chapple, 2011). In addition, this pathway helps the plant survive and adapt to new habitats (Bais et al., 2003), and provides metabolites for plant reproduction (Dudareva et al., 2004). The pathway begins with three reactions leading to the synthesis of 4-coumaroyl CoA (alternatively, p-coumaroyl CoA) (Fraser and Chapple, 2011), from which various secondary metabolites are generated (Figure 1.9).

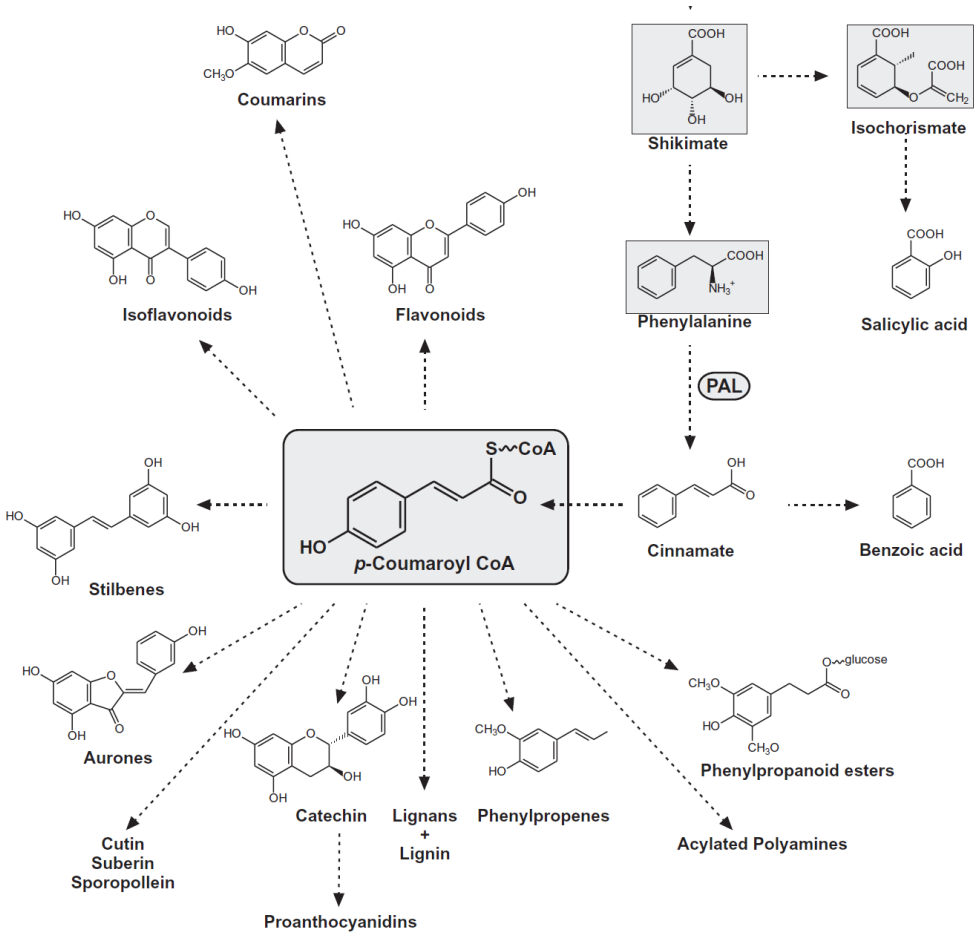


Figure 1.9. The general phenylpropanoid pathway in plants (Vogt, 2010).

The phenylpropanoid pathway is initiated by the action of phenylalanine ammonia lyase (PAL) as the first key enzyme that converts an end product of the shikimate pathway, phenylalanine, to cinnamic acid, the branch point enzyme between primary shikimate pathway (Herrmann, 1995) and secondary (phenylpropanoid) metabolism (Hahlbrock and Scheel, 1989, Dixon et al., 1992). PAL enzyme is encoded by multiple genes. However, it is not clear whether the size of this gene family is due to the organisation of different forms of the enzyme in metabolic compartments or it simply allows for increased enzyme production under stress conditions (Dixon and Paiva, 1995). Many PAL genes have been identified and characterised in some plant species, such as Arabidopsis (4 genes), tobacco (4 genes), poplar (5 genes), bean (3 genes), rice (12 genes), potato (40-50 genes), maize (20

genes) and cassava (3 genes) (Hamberger et al., 2007, Liang et al., 1989, Beeching et al., 2000, Reichert et al., 2009, Huang et al., 2010, Rosler et al., 1997, Kong, 2015). Many of which are induced in response to wounding or to feeding by herbivores (Dixon and Paiva, 1995). For instance, all three bean PAL genes are activated by wounding, whereas only PAL1 and PAL3 are induced by fungal attack (Liang et al., 1989) and PAL genes from cassava are activated by wounding leading to the production of coumarins, including scopoletin that triggers blue-black vascular streaking or PPD in cassava.

1.5. Biosynthesis of scopoletin in cassava and its role on PPD

Scopoletin (6-methoxy-7-hydroxy-coumarin), one of the phenylpropanoid metabolism products belong to coumarins, is considered as a major component, together with ROS, and contributes to the blue-black discoloration of the cassava root, observed as PPD. Bayoumi *et al* (2008) studied the three likely alternative scopoletin biosynthetic pathways via ferulate (1) hydroxycinnamate (2), and hydroxycaffeate intermediates (3), of which that via the ferulate intermediate is the major pathway leading to the biosynthesis of scopoletin in cassava (Figure 1.10). This pathway is also supported in *Arabidopsis thaliana*, where a member of the Fe(II)- and 2-oxoglutarate-dependent dioxygenase (2OGD) family, named F6'H1, plays a key role in the biosynthesis of scopoletin (Matsumoto et al., 2012, Kai et al., 2008).

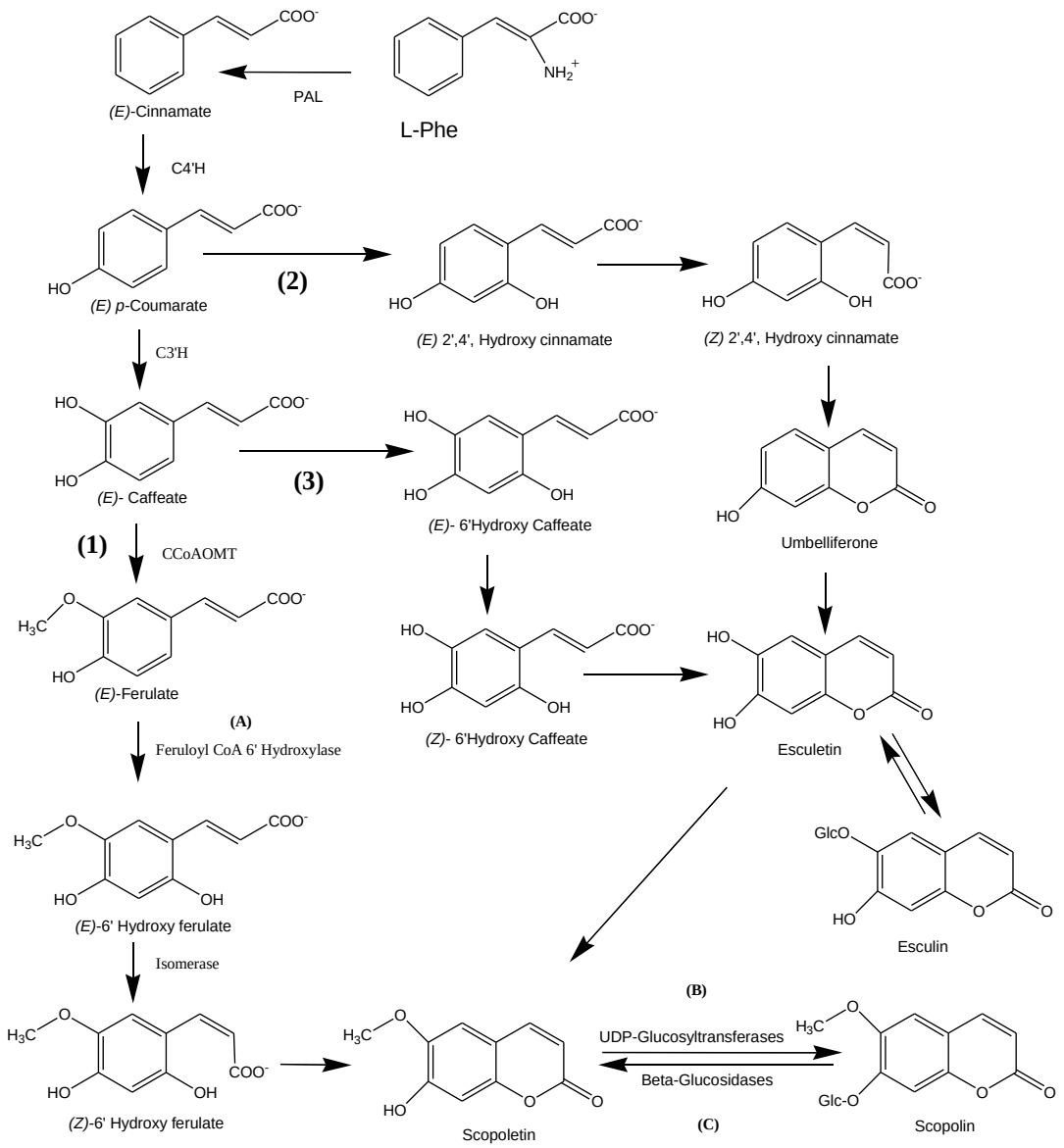


Figure 1.10. Biosynthesis of scopoletin and scopolin in cassava. The figure was re-drawn and slightly modified from (Bayoumi et al., 2008).

The accumulation of scopoletin is associated with the response to microbial attack or other stress including physical injury and dehydration (Tanaka et al., 1983, Buschmann et al., 2000, Bayoumi et al., 2010, Chong et al., 2002, Gachon et al., 2004). Scopoletin accumulates rapidly during PPD. It is usually present at trace amounts to undetectable levels in the fresh cassava root and its accumulation is detected shortly and significantly after injury, peaking at around day 2, depending on genotypes (Bayoumi et al., 2010,

Buschmann et al., 2000). These data imply that scopoletin plays an important role in the wound response. Peroxidase-mediated oxidation of scopoletin forms a blue-black complex (Miller et al., 1975), which is strongly correlated with the blue-black discoloration of the roots. Application of exogenous scopoletin to fresh cassava roots produced more intense fluorescence under UV light and more rapid discoloration (Wheatley and Schwabe, 1985), providing further evidence that scopoletin is one of the key components in the PPD response. In addition, scopoletin has antibacterial and antifungal activities (Gnonlonfin et al., 2012). Like many bioactive molecules, scopoletin is generally found in a modified non-active form as its glycone, scopolin. Whilst some scopoletin is synthesised *de novo*, it is also released from its inactive glycone, scopolin, via de-glucosylation by the action of specific beta-glucosidase.

In plants, scopoletin and scopolin are interconverted by two enzymes: a scopoletin-glucosyltransferase (scopoletin-GT) and a scopolin-beta-glucosidase (scopolin-BG). In cassava, the genes for these enzymes have not been well studied. On the other hand, they have been identified in other plants, such as tobacco and *Arabidopsis*. There is strong evidence that inhibiting the expression of the genes responsible for the interconversion of scopoletin and scopolin will reduce levels of these compounds. Down regulation of a UDG-Glc:phenylpropanoid glucosyltransferase in tobacco through the expression of an anti-sense construct led to the reduced accumulation of both scopoletin and scopolin, amongst other effects (Chong et al., 2002). While the overexpression of a scopoletin-glucosyltransferase in tobacco led to the two-fold higher accumulation of scopoletin and scopolin in response to viral infection (Gachon et al., 2004). Enzymes involved in the hydrolysis of scopolin in the roots of *Arabidopsis* have been studied through their expression in insect cells providing further insights into their mode of action and the roles of accessory proteins (Ahn et al., 2010). These findings suggest that the study of the corresponding genes in cassava and analysis

of their function on the accumulation of scopoletin and scopolin could provide clues as to the effects of these compounds on the root deterioration process.

1.6. Wound stress responses of other root and tuber crops

Storage roots and tuberous organs are often damaged during harvest, transportation and storage. In response to wounding, cassava show common responses to those of other root and tuber crops such as sweet potato, yam, taro (aroids), and potato, but with some unique and distinctive features.

1.6.1. Sweet potato

Sweet potato *(Ipomoea batatas* (L.) Lam., Convolvulaceae, Dicotyledons) is the seventh's world crop after cassava. It is native to the American continent and mainly cultivated for its storage root, as is cassava. Sweet potato is now widely cultivated across tropical Africa, Asia and America, in more than 100 countries, and is utilised as both a food and industrial crop (Lebot, 2009). In sweet potato, injury was normally cured by the formation of barrier layers of lignin and suberin at the wounded sites. Upon tissue disruption or injury, PAL activity increases in wounded tissue shortly after injury and reaches a peak within 24 h, after which its activity gradually decreased (Tanaka and Uritani, 1977, Tanaka et al., 1974). The activation of PAL enzymes leads to the production of polyphenols such as chlorogenic acid, isochlorogenic acid and the relatives in the tissue near the wounded or infected region of sweet potato (Uritani, 1999), which were significantly higher compared to those detected in cassava (Akazawa and Uritani, 1961, Tanaka et al., 1983). Peroxidase which is involved in the biosynthesis of lignin was also activated in response to wounding (Uritani, 1999), this enzyme is most likely associated with the curing process. The rate of curing or healing is dependent on the storage conditions. Storing the harvested roots at 30-35 °C and high humidity (80-90%) for 4-7 days accelerated the

formation of toughened periderm (Lebot, 2009). Interestingly, coumarins were neither present in fresh sweet potato nor wounded tissue. These compounds were only detected in infected tissue in response to fungal infection (Uritani, 1999).

1.6.2. Yam

Cultivated yams (*Dioscorea* spp., Dioscoreaceae, Monocotyledons) are grown for their storage roots, which provide a staple food for people in the tropical countries of Africa (particularly West Africa), South America, Asia and the Pacific. In response to wounding, the roots produce a barrier layer of cork periderm at the wound site within in five days (Ravi et al., 1996), which is accompanied by the accumulation of polyphenols 5-6 fold higher than measured in fresh tubers (Ikediobi et al., 1989). A common wound response identified in yams is the activation of oxidation-related enzymes such as polyphenol oxidase, peroxidase, and lipoxygenase, with polyphenol oxidase becomes the most significant enzyme up-regulated, suggesting the importance of the enzyme in stress response (Ikediobi et al., 1989).

1.6.3. Taro (Aroids)

Taro, *Colocasia esculenta* (L.) Schott (Araceae, Monocotyledons), is an ancient crop and is probably the oldest crop on this planet that has been cultivated in tropical Asia for more than 10,000 years (Lebot, 2009). Like sweet potato, taro also produces a lignin layer adjacent to the wounded tissue when it is wounded. Polyphenols such (+) catechin and its derivates were detected in the wounded root of taro, which is similar to the cassava response (Tanaka et al., 1983). No PAL enzyme activity was reported in taro upon injury and neither did coumarins accumulate (Uritani, 1999). However, an increase in PAL activity would have been expected since lignin and polyphenols were identified in the wounded roots.

1.6.4. Potato

Potato, *Solanum tuberosum* L., is the world's fourth-largest food crop in the world after maize, wheat and rice and is grown for its starchy tuber. Potato becomes responsive to gibberellins after wounding leading to enhanced RNA synthesis (Wielgat and Kahl, 1979). Like other crops, potatoes have shown a number of responses to wounding including the accumulation of phytoalexins, lignin or lignin-like material, callose, extensins, glycine-rich proteins, suberin and proteinase inhibitor, as well as increase in the enzymatic activities of chitinase, glucanase, phenylpropanoid and terpenoids pathways (Rumeau et al., 1990). In response to wounding, callose was produced in the first 15 min after injury at the site of plasmodesmata and sieve plate pores (Thomson et al., 1995) leading to the synthesis of ethylene (Aked, 2001). Ethylene stimulates the pathway of phenolic-acid biosynthesis leading to lignin formation. Several enzymes were activated in response to wounding such as lipoxygenase (Lulai and Corsini, 1998) and two plant defense genes: extensin and PAL. There was a significant increase in the activity of tyramine hydroxycinnamoyl transferase (THT) within 3-4 h after wounding. This enzyme catalyses the synthesis of hydroxycinnamoyl tyramines which are associated with the formation of suberization (Negrel et al., 1993). Starch degradation was observed in the first 12 h after injury to release glucose via an increase in alpha and beta-amylases activity (Kato and Uritani, 1976).

In summary, some crops such as cassava, sweet potato, yam, taro and potato, demonstrate similar responses to wounding including the activation of PAL enzyme and the production of various secondary metabolites such as polyphenol compounds, suberin and lignin. The latter two compounds play roles in wound healing. Despite, their commonality in wound responses, they also have shown some differences and unique response characteristics. For example, production of polyphenol in cassava is also less compared to sweet potato and formation of lignin in cassava is much

slower than sweet potato and taro that lead to impaired wound repair. However, it only occurs when cassava roots are detached from the main stem, which could suggest that there are components required for healing that are not present in the roots alone. The major biological differences between cassava and these roots/tubers is that cassava is not a propagule. Cassava root is anatomically a true root not a tuberous (Alves, 2002) like potato, therefore, inadequate wound healing in detached cassava root could be related to the fact that it no longer has a biological function and, as a result, there is no need for the storage roots to repair their wounds. When the roots are still attached to the stem, the wounding is completely cured.

1.7. Genetic transformation in cassava

Cassava improvement through conventional breeding is hampered due to the high levels of genetic heterozygosity, poor flowering, low pollen fertility, self-incompatibility, and low fruit set (Ceballos et al., 2004). On the other hand, genetic transformation has shown to be the most effective mechanism to introduce or manipulate genes regulating important traits in cassava within a reasonable timeframe. Therefore, in this study we used genetic transformation approach to study cassava PPD-related genes.

Recent progress in cassava transformation has produced a robust and high-throughput protocol for the production and analysis of transgenic cassava plants via Agrobacterium-mediated transformation of friable embryogenic calli (FEC) (Bull et al., 2009, Zainuddin et al., 2012, Taylor et al., 2012). Agrobacterium-mediated transformation of FEC has been widely used over the last decade. There are several advantages of using this method: first is that using FEC as starting material reduces the risk of generating chimeric plant compared to organised tissues such as cotyledons. Second, this system would produce fewer non-transformed plantlets in the antibiotic resistance selection (Gonzalez et al., 1998, Zhang et al., 2000, Schreuder et al., 2001). Despite all the advantages, this method remains a tedious and

labour-intensive procedure, which can take about 6 months to regenerate plantlets from FEC (Bull et al., 2009) if materials used and all steps go according to plan. Drawbacks and difficulties are mainly due to the low regeneration of plantlets from somatic embryos (Baba et al., 2008) and highly variable numbers of transgenic events (Koehorst-van Putten et al., 2012). As a result, cassava transformation requires well-trained tissue culture specialist, substantial quantity and quality of FEC material and transformation repetition in order to generate a sufficient number of independent transgenic lines (Zainuddin et al., 2012).

Chapter 2. Materials and Methods

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2.1. Plant materials

2.1.1. Cassava

Sample of cassava leaves and roots used in the beginning of this study were obtained from wild type West African cultivar (cv) 60444 grown in the glasshouse at the University of Bath under controlled conditions of 25-30 ºC, > 50% relative humidity, and 16 h daylight. Whilst some cassava roots were purchased from Morrison's supermarket located in London Road, Bath.

2.1.2. Friable Embryogenic Callus (FEC) of cassava

FECs of cassava cv. 60444 used for cassava transformation, which were induced from shoot cultures, were kindly provided by Dr. Ima Zainuddin, ETH-Zurich, Switzerland. FECs and transformed FECs were cultured and maintained on Gresshoff & Doy (GD) agar plate in a controlled room or growth chamber at 28 ºC, 16 h light/ 8 h dark (Zainuddin et al., 2012).

2.1.3. Cassava *in vitro* culture

Transgenic and non-transgenic plants generated from FECs of cassava cv. 60444 were propagated *in vitro* in cassava basic medium (CBM) containing Murashige & Skoog (MS) medium supplemented with 2% (w/v) sucrose. *In vitro* cultures were propagated and maintained in the growth room at the University of Bath at 25-28 ºC, > 50% relative humidity and 16 h daylight. Propagation of *in vitro* culture was done every 4-5 weeks depending on the purpose of the experiment. For maintenance of the *in vitro* culture collection, it is normally done every 6 months.

2.1.4. Arabidopsis

Wild-type Arabidopsis used in this study was *Arabidopsis thaliana* ecotype Columbia-0, obtained from Dr. Yaxiao Li, University of Bath. Seeds of Arabidopsis T-DNA insertion lines (Table 2.1) were purchased from The

Nottingham Arabidopsis Stock Centre (NASC). Soil-grown Arabidopsis for genotyping analysis, and plant transformation and MS agar-grown *in vitro* plants for biochemical analysis were grown in the growth room at University of Bath under controlled conditions of 21-22 °C, 50-60% relative humidity, and 16 h daylight.

Table 2.1. List of Arabidopsis T-DNA insertion lines.

No	T-DNA line	Gene ID	Gene family	Functional annotation
1	SALK_097487	At4g34131	UDP-glucosyltransferase	Transferase activity
2	GK-732F07	At3g09260	Glycosyl hydrolase family 1	β-glucosidase activity

2.2. Antibiotics for bacterial and transgenic line selection

All antibiotics (Table 2.2) were purchased from Sigma Aldrich, UK unless otherwise stated. Each antibiotic was dissolved in its appropriate solvent, filter-sterilised using 0.22 µm Millipore filter (Millex Ltd.) and stored in 1 mL aliquots. All stock solutions were stored at -20 ºC until use.

Table 2.2. List of antibiotics

No	Antibiotic	Solvent	Stock solution (mg mL^{-1})	Company	Usage
1	Ampicillin	Distilled Water	100		Bacterial selection
2	Kanamycin	Distilled Water	50		Bacterial selection
3	Zeocin™		100	Invitrogen	Bacterial selection

4	Gentamycin	Distilled Water	50	Bacterial selection
5	Rifampicin	Methanol	50	Bacterial selection
6	Streptomycin	Distilled Water	100	Bacterial selection
7	Carbenicillin	Distilled Water	500	Bacterial selection
8	Hygromycin	Distilled Water	50	Transgenic plant selection

2.3. Plasmids/Vectors

Several vectors were used in plasmid constructions including pUC19 (New England Biolabs (NEB)), pGEM®-T Easy vector system I (Promega), pKANNIBAL (CSIRO, Australia), pDONR/Zeo (Invitrogen), and binary vector pCAMBIA 1305.1 (GenBank: AF354045, http://www.cambia.org) that had been modified by Page (2009), University of Bath. Modification of pCAMBIA 1305.1 was first made to replace the GUS reporter gene region with Gateway cassette (Figure 2.1), in order to permit Gateway cloning. The second modification was to replace 35S promoter with root-specific from potato *StPATATIN* promoter shown in figure 2.2 (Page, 2009). All other maps of plasmids used in this study are available in Appendix 1. Additional information on the plasmids regarding its appropriate antibiotic selection is shown in table 2.3.

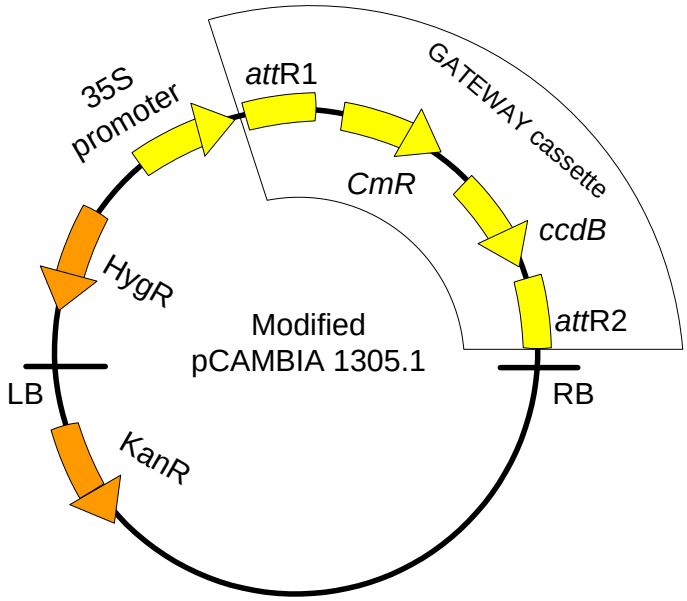


Figure 2.1. Modified pCAMBIA 1305.1::35S//Gateway cassette used as an expression vector in plasmid construction.

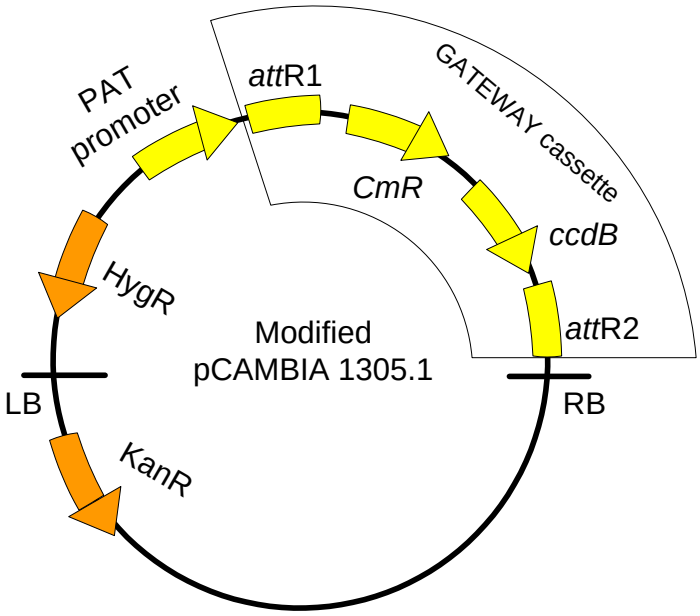


Figure 2.2. Modified pCAMBIA 1305.1::StPAT//Gateway cassette used as an expression vector.

Table 2.3. List of vectors for plasmid constructions.

No	Name of Plasmid/Vector	Company/source	Antibiotic concentration required (mg L^{-1})
1	pUC19	NEB	Ampicillin 100
2	pKANNIBAL	CSIRO Plant Industry, Australia	Kanamycin 50
3	pDONR/Zeo	Invitrogen	Zeocin 50
4	pGEM®-T Easy	Promega	Ampicillin 100
5	pCAMBIA 1305.1	The Centre for the Application of Molecular Biology to International Agriculture (CAMBIA, http://www.cambia.org	Kanamycin 100
6.	Modified pCAMBIA 1305.1::gateway cassette	Page, 2009	Kanamycin 100
7	Modified pCAMBIA 1305.1::gateway cassette-*StPATATIN*	Page, 2009	Kanamycin 100

2.4. Restriction Endonucleases

Various restriction endonucleases or restriction enzymes (RE) were used in the preparation of gene constructs as well as analysis of transgenic cassava plants. Details of RE used in this study are available in table 2.4 below.

Table 2.4. List of restriction enzymes.

No	Restriction enzymes	Stock concentration (U/µL)	Company	Application
1	*Xho*I	10	Promega	Hairpin-RNAi construct

2	*Cla*I	10	Promega	Hairpin-RNAi construct
3	*Xba*I	10	Promega	Hairpin-RNAi construct
4	*Kpn*I	10	NEB	Hairpin-RNAi construct
5	*Pst*I	20	NEB	Linearise PGEM vector
6	*Hind*III	20	NEB	Digest cassava genomic DNA (Southern blotting)

2.5. Oligonucleotides (primers) design

Many primers pairs were designed for various purposes such as PCR detection, PCR genotyping, (*att*achment) *att*B site-PCR, colony PCR, DNA sequencing, and RT-qPCR. Primers design was done using bioinformatics tools listed in section 2.6 and the primers were ordered from Fisher Scientific Institute. A list of all primers and their application is given in table 2.5 with Tm (ºC) calculated using online software, OligoAnalyzer3.1.

Table 2.5. List of primers and its application.

Primer name and application	**Primer sequence (5'→ 3')**	**Tm (ºC)**
2.5.1. Gene specific primers for cassava gene detection and expression		
Cassava4.1_023510_FWD	GTGTTCCTGCCTGTCATGAC	56
Cassava4.1_023510_REV	GTGATGGAATCCATGTTCTCGC	56.1
Cassava4.1_025417_FWD	AGAGAAGGCAATCACTGAATTG	53.1
Cassava4.1_025417_REV	ACCTTCTTCAACAGCTTGTG	53.1
Cassava4.1_006629_FWD	CCCAGTAAACGCAACTCGT	55.3
Cassava4.1_006629_REV	TGAAACGAGACCATGTGGACA	56.4
Cassava4.1_030942_FWD	GGCCACACTATTCCCCTCA	56.8
Cassava4.1_030942_REV	AGCTCTTCGAGCGGTTGAG	57.3
Cassava4.1_029076_FWD	TCACCACTCCTCTCAACTCG	56.1
Cassava4.1_029076_REV	AGGCTCTTGAAGCATGGATACT	56
Cassava4.1_029292_FWD	CCCCTGTAAACGCACCTTG	56.3
Cassava4.1_029292_REV	AGCTAGTACCACTGAAGGACAG	55.7
Cassava4.1_031067_FWD	GTTATAGCACCACTGGCTATAACAC	55.3
Cassava4.1_031067_REV	TTAGAATTAGCAGGTAAAGCAGCTG	55.2

Cassava4.1_021061_FWD	CATCTAAGCACTCTCTTCACCTG	56.9
Cassava4.1_021061_REV	TAATAGAAACCATCCGCAACGTC	55.2
Cassava4.1_008910_FWD	TATCTCCTGGCCTAGAATTTTGC	54.7
Cassava4.1_008910_REV	CTTCAAGGGCTTGTGGAACA	55.5
Cassava4.1_029389_FWD	GATTCCAACCTTATCTTCACAGCTC	55.5
Cassava4.1_029389_REV	CTCCTGCACTGACTCTTCC	54.9
2.5.2. Amplification of full-length cDNA of Arabidopsis gene, At3g09260 (BGLU23)		**Tm (ºC)**
At3g09260_Fwd Primer3	ACAAACAAAAATGGTTTTGCAAAAGCTTC	56.8
At3g09260_Rev Primer3	CAGAAATAGCTTAAAGCTCATCCTTCTTG	56
2.5.3. Amplification of full-length cDNA of targeted cassava genes		**Tm (ºC)**
Cassava4.1_006629FcDNA_FWD3	CCAAATCTGAAACATGTGCTCTG	54.6
Cassava4.1_006629FcDNA_REV3	AGTAATTGTTGCAGGCCTTAAG	53.3
Cassava4.1_031067FcDNA_FWD3	CTTGCCGTAATGGCAAATGAAG	55
Cassava4.1_031067FcDNA_REV3	GCATAATTGGTCTTATCTCTGGAG	53.1
Cassava4.1_021061 FcDNA_FWD3	CACTATGGGTATGGCATCTAAGC	55.2
Cassava4.1_021061 FcDNA_REV3	AGGACAACACGAGCTACATTATG	54.7
Cassava4.1_008910 FcDNA_FWD2	GTGGCTCTAATGAAAGGATTAGG	53.1
Cassava4.1_008910 FcDNA_REV2	GTTCCTCATCCTATGCTGTGTG	55.4
Cassava4.1_029389 FcDNA_FWD2	GTAGGCAAATAATGAAAATTCAACC	51.3
Cassava4.1_029389 FcDNA_REV2	TAGTGACATTTTCCCCATTGAG	52.6
2.5.4. Amplification of RNAi target region		**Tm (ºC)**
Cassava4.1_006629B_Fwd Primer1	GAGTGTCTGAAATGGCTTGATGCG	58.5
Cassava4.1_006629B_Rev Primer1	CCGGCACTCACACCTTCTAAAGT	59.1
2.5.5. T-DNA insertion lines genotyping		**Tm (ºC)**

SALK_040045_LP	GGTTACGCAAGTGCTCAGAAC	56.4
SALK_040045_RP	TGGATGTCGATTTCGAAACTC	52.8
SALK_097487_LP	GATATGCCACGCTCTCTTCAG	55.4
SALK_097487_RP	CCGTAAATGGCAAAACAAAAG	51
SALK_LB.1.3	ATTTTGCCGATTTCGGAAC	51.5
SAIL_156LP	CCGTGCAAGATTGTTCAAGAC	54.5
SAIL_156RP	GCTTAAAAAGCCGCCATATTC	52.6
SAIL_LB1	GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC	61
GABI_732F07LP	ATCACTTTCAATGAGCCATGG	53.3
GABI_732F07RP	AACAGAATCTGAGGCCCCTAG	56.2
GABI_08474_LB	ATAATAACGCTGCGGACATCTACATTTT	56.9
2.5.6. Construction of overexpression vector of At3g09260 (BGLU23)		**Tm (ºC)**
At3g09260_12*att*B1_FWD	AAAAAGCAGGCTACAAACAAAAATGGTTTTGCAAAAGCTTC	63.1
At3g09260_12*att*B2_REV	AGAAAGCTGGGTCAGAAATAGCTTAAAGCTCATCCTTCTTG	63.9
*att*B1 adapter_FWD	GGGGACAAGTTTGTACAAAAAAGCAGGCT	61.4
*att*B2 adapter_REV	GGGGACCACTTTGTACAAGAAAGCTGGGT	63.7
2.5.7. Construction of RNAi vector (Cassava4.1_006629 = *MeSGT*1)		
Sense orientation fragment		**Tm (ºC)**
Cassava4.1_006629S_12*att*B1_FWD	AAAAAGCAGGCTGAGTGTCTGAAATGGCTTGATGCG	65.8
Cassava4.1_006629S_Acc65I_REV	CTCGTTGGTACCCCGGCACTCACACCTTCTAAAGT	67
Cassava4.1_006629S_XhoI-*att*B1_FWD	GATGCTCGAGGGGGACAAGTTTGTACAAAAAAGCAGGCT	67
Anti-sense orientation fragment		**Tm (ºC)**
Cassava4.1_006629AS_12*att*B2_FWD	AGAAAGCTGGGTGAGTGTCTGAAATGGCTTGATGCG	66.5

Cassava4.1_006629AS_ClaI_REV	TCGTATCGATCCGGCACTCACACCTTCTAAAGT	64.2
Cassava4.1_006629AS_XbaI-*att*B2_FWD	ATGCTCTAGAGGGGACCACTTTGTACAAGAAAGCTGGGT	66.9
2.5.8. Gene expression in the Arabidopsis knock-out lines		**Tm (ºC)**
At3g09260GSP_FWD	GCAGTTGAAGCTTACCGGAAATG	56.7
At3g09260GSP_REV	ATGATCAGCGGTACCAACAG	54.8
At4g34131GSP_FWD	CGACTTTTACAAGAGTGTTGTACTG	53.9
At4g34131GSP_REV	AGATCGTTGAAAGAAGACCCTC	54.3
2.5.9. DNA sequencing		**Tm (ºC)**
M13_FWD (-20)	GTAAAACGACGGCCAGT	52.6
M13_REV (-24)	AACAGCTATGACCATG	45.4
At3g09260GSPF1_FWD	ATGGTTTTGCAAAAGCTTCCTC	54.5
At3g09260GSPF1_REV	CTCGGAAATCCTTCACAATCC	53.4
At3g09260GSPF2_FWD	GAAAGGATTGTGAAGGATTTCCGAG	55.9
At3g09260GSPF2_REV	GCATCCATCTTGGTTTTGAAGGATC	56.5
At3g09260GSPF3_FWD	GGAGAAACCTGATCCTTCAAAACC	55.9
At3g09260GSPF3_REV	TTAAAGCTCATCCTTCTTGAGCG	55.2
pKan_35S_SeqFWD	ACGCACAATCCCACTATCCTTC	57
PDK_Intron_Fwd primer	TGCTAATATAACAAAGCGCAAGAT	53.1
pKan_OCS_SeqREV	GTAAGGATCTGAGCTACACATGCTC	56.9
pKan_629S_FwdSeq	GAAGACGTTCCAACCACGTC	56.1
pKan_629S_RevSeq	GTTACCTTGTTTATTCATGTTCGAC	52.5
pKan_629AS_FwdSeq	CAAGTGATGTGTAAGACGAAGAAG	53.6

pKan_629AS_RevSeq	AAGGATCTGAGCTACACATGC	54.6
PGEM_T7_FWD	TAATACGACTCACTATAGGG	47.5
PGEM_SP6_Rev	CATTTAGGTGACACTATAG	44.2
M13 Forward (-20) pDNOR	GTAAAACGACGGCCAG	50.7
M13 Reverse pDNOR	CAGGAAACAGCTATGAC	47
pCAMBIA_35S Fwd primer	TGACGTAAGGGATGACGCAC	57.2
pCAMBIA_OCS Rev Primer	TAATCATCGCAAGACCGGCA	56.8
pCAMBIA_PAT Fwd primer	GCGGTGCAAAGTGAGTGAGA	57.9
2.5.10. Analysis of cassava transgenic lines		**Tm (ºC)**
Hygromycin-based Fwd primer	TCTCGATGAGCTGATGCTTTGG	57.2
Hygromycin-based Rev primer	AGTACTTCTACACAGCCATCGG	56.2
Cassava4.1_006629 qPCR F1	AGGCTCATAGAGGAAGGGAAGC	58.4
Cassava4.1_006629 qPCR R1	TGGGTTTCTTCGCATCAAGCC	58.3
Cassava4.1_006629 qPCR F2	AGGCTCATAGAGGAAGGGAAGC	58.4
Cassava4.1_006629 qPCR R2	GCCCTATTGCAATCTCATGTAGC	56.1
Cassava4.1_006629 qPCR F3	CTCACTGTGGGTGGAACTCTAC	56.9
Cassava4.1_006629 qPCR R3	TGGTAATCCGGCACTCACAC	57.3
At3g09260 F1 qPCR	TGCGGCATACCAGGTCGAAG	59.9
At3g09260 R1 qPCR	GATGTCCCATAAGGCTGGTCC	57.5
At3g09260 F2 qPCR	GGACCAGCCTTATGGGACATC	57.5
At3g09260 R2 qPCR	CGATGGAAGAAATCAACGGCC	56.6
At3g09260 F3 qPCR	GAATGTCTATCGCATGGCCAAG	56.2
At3g09260 R3 qPCR	CACACCAGCTTGACTCACTCC	57.9
PP2A housekeeping gene FWD	TGCAAGGCTCACACTTTCACT	57.1
PP2A housekeeping gene REV	CTGAGCGTAAAGCAGGGAAG	55.8
Ubq10 housekeeping gene FWD	CACCGGATCAGCAAAGGCTTA	60
Ubq10 housekeeping gene REV	CAGACACACAGATCAAAGCAGC	60

2.6. Bioinformatics tools

Online software and database including The National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) and Plant Genomic Resource, Phytozome (http://www.phytozome.net/search.php) were used to search for homologous genes in target plants. The Carbohydrate-Active enZYme (CAZY) database (http://www.cazy.org/Welcome-to-the-Carbohydrate-Active.html) and a database for Plant Carbohydrate-Active enzyme (PlantCAZyme) (http://cys.bios.niu.edu/plantcazyme/index.php) were used to identify gene family of identified homologous cassava genes. Online software from Salk Institute Genomic Analysis Laboratory (SIGnAL), http://signal.salk.edu/cgi-bin/tdnaexpress?gene= was used to identify Arabidopsis knock out lines for functional studies. Bioinformatics software, Geneious v5.3 (Kearse et al., 2012) was used to align nucleotide or amino acid sequences, analyse phylogenetic tree, design primers, and identify restriction sites within gene of interest or vector. Online software, http://eu.idtdna.com/calc/analyzer was also used for primer design. Matlab-based imaging software was used to assess PPD score of cassava root (Vanderschuren et al., 2014). SigmaPlot 10.0 software and Excel were used for statistical analysis. ChemDraw Ultra 8.0 was used to re-draw chemical structures.

2.7. Bacterial strains

2.7.1. *Eschericia coli*

Commercial competent *E. coli* cells used in *E. coli* transformation were 10-beta competent *E. coli* (High Efficiency) strain K12 (New England Biolabs (UK), Ltd).

2.7.2. *Agrobacterium tumefaciens*

Homemade competent *A. tumefaciens* cells used in cassava transformation were prepared from wild-type *A. tumefaciens* strain LBA4404::pAL4404 (Hellens et al., 2000), which was generous gift of Dr. Herve Vanderschuren

and Dr. Ima Zainuddin, ETH-Zurich, Switzerland. While, homemade competent *A. tumefaciens* cells used in Arabidopsis transformation were prepared from wild-type *A. tumefaciens* strain GV3101::pMP90 (pTiC58) (Hellens et al., 2000), which was obtained from Dr. Yaxiao Li, University of Bath, UK. The cultures of *E. coli* and *A. tumefaciens* were maintained in 50% (v/v) of glycerol solution and stored at -70 °C until use.

2.8. Media for bacterial growth

All chemicals were obtained from Sigma Aldrich, UK unless otherwise stated. All media was dissolved in distilled water (DW), sterilised by autoclaving at 121 °C for 20 min. unless otherwise stated.

2.8.1. SOC broth

S.O.C is Super Optimal Broth (SOB) with addition of sucrose and used in *E. coli* transformation. The S.O.C medium consisted of (per L) 20 g of Bacto™ Tryptone (Scientific Laboratory Supplies (SLS)), 5 g of Yeast Extract, 0.6 g of NaCl (10 mM), 0.2 g of KCl (2.5 mM), 0.95 g of $MgCl_2$ (10 mM), 2.5 g of $MgSO_4.7H_2O$ (10 mM) and 20 mM sucrose and pH 7.0.

2.8.2. LB broth

LB broth was prepared from (per L) 10 g of Bacto™ Tryptone (SLS), 10 g of NaCl and 5 g of Yeast Extract and pH 7.0.

2.8.3. LB agar

LB agar was prepared from LB broth (2.8.2) with addition of 15 g of agar bacteriological No. 1 (Oxoid). After autoclaving, about 25 mL of agar medium was poured into round-bottom petri dish (90 mm diameter).

2.8.4. Low salt LB

Low salt LB broth was prepared from (per L) 10 g of Bacto™ Tryptone (SLS), 5 g of NaCl and 5 g of Yeast Extract and pH 7.5.

2.8.5. 2YT broth

Growth medium for *A. tumefaciens,* 2YT broth was prepared from (per L) 16 g of Bacto™ Tryptone (SLS), 5 g of NaCl and 10 g of Yeast Extract and pH 7.0. LB medium was also used for *A. tumefaciens.*

2.9. Media for plant growth

2.9.1. MS agar for Arabidopsis growth

Murashige & Skoog (MS) agar consisted of (per L) 4.4 g of MS powder including Gambrong B5 vitamin (Duchema Biochemie), 10 g of Sucrose and 0.5 g of MES, 2-(N-morpholino)ethanesulfonic acid. pH was adjusted to 5.9 and 8 g of phytoagar (Duchema Biochemie) was added.

2.9.2. Soil medium for Arabidopsis growth

Soil medium for Arabidopsis was prepared from NPK fertilizer, F2S (Fine+Sand) containing N144-P73-K239 (Scotts Levington). It was poured into a tray consists of 40 cells or different type of tray depending on purpose of the experiment, with capillary mat placed underneath the cells. Instead of autoclaving media, 0.2 g/L of insecticide, intercept 70WG (a product of Bayer Germany), was added to sterilise the soil.

2.9.3. Cassava Basic Medium (CBM) agar for cassava growth

CBM was used for cassava *in vitro* propagation and maintenance. It was prepared from (per L) 4.4 g of MS powder including vitamin (Duchema Biochemie), 20 g of sucrose, 100 μL of 20 mM $CuSO_4$ (2 mM final concentration), 3 g of gelrite (Duchema Biochemie) and pH 5.8.

2.9.4. Soil medium for cassava growth

Soil medium for cassava was prepared by mixing up NPK fertilizer, M2 containing N192-P98-K319 (Scotts Levington) with perlite with ratio of 3:1 (w/w). The soil was then poured into a tray or pot as needed with no need for sterilization.

2.10. Media for plant transformation

2.10.1. Arabidopsis transformation

2.10.1.1. LB liquid medium for *A. tumefaciens* strain GV3101::pCAMBIA 1305.1

LB liquid medium (2.8.2) supplemented with (per L) 25 mg of Gentamycin, 30 mg of Rifampicin and 50 mg of Kanamycin.

2.10.1.2. LB agar for *A. tumefaciens* strain GV3101::pCAMBIA 1305.1

LB agar (2.8.3) supplemented with (per L) 25 mg of Gentamycin, 30 mg of Rifampicin and 50 mg of Kanamycin.

2.10.1.3. Solution buffer for Arabidopsis transformation

Solution buffer consisted of (per L) 50 g of sucrose (5%) and 2 g of $MgCl_2$. No autoclave required.

2.10.1.4. LB-H25 agar for selection of transgenic lines

LB agar (2.8.3) added with (per L) 25 mg of Hygromycin as plant selection marker.

2.10.2. Cassava transformation

Protocol for cassava transformation was adopted from Bull et al (2009) with some modifications.

2.10.2.1. Gresshoff & Doy (GD) agar

GD agar medium for induction and propagation of FEC consisted of (per L) 2.7 g of GD medium including vitamins (Duchema Biochemie), 20 g of sucrose and 12 mg of picloram (Duchema Biochemie) with 8 g of Noble agar (Difco), pH 5.8.

2.10.2.2. LB agar medium for *A. tumefaciens* strain LBA4404::pCAMBIA 1305.1

LB agar (2.8.3) contained (per L) Streptomycin 100 mg, 25 mg of Rifampicin and 50 mg of kanamycin.

2.10.2.3. LB liquid medium for *A. tumefaciens* strain LBA4404::pCAMBIA 1305.1

LB liquid medium (2.8.2) contained (per L) Streptomycin 100 mg, 25 mg of Rifampicin and 50 mg of kanamycin.

2.10.2.4. GD solution (GDS) for Agrobacterium preparation

GDS consisted of (per L) 2.7 g of GD medium including vitamins, 20 g of sucrose and 12 mg of picloram (Duchema Biochemie) and pH 5.8.

2.10.2.5. GD-C500 for washing of transformed FEC

GD-C500 was prepared from GDS (2.10.2.4) added with 500 mg/L of carbenicillin.

2.10.2.6. GD-C250 agar for recovery of transgenic FEC

GD-C250 was prepared from GDS (2.10.2.4) added with 250 mg/L of carbenicillin.

2.10.2.7. Media for maturation of transgenic FEC

There are three compositions of media for this purpose:

a. GD-C250-H5 was prepared from GD-C250 (2.8.4.6) supplemented with 5 mg/L of hygromycin
b. GD-C250-H8 was prepared from GD-C250 (2.8.4.6) supplemented with 8 mg/L of hygromycin
c. GD-C250-H15 was prepared from GD-C250 (2.8.4.6) supplemented with 15 mg/L of hygromycin.

2.10.2.8. MSN-C250-H15 agar for regeneration of transgenic embryos

MSN agar was prepared from (per L) MS agar with addition of 250 mg of carbenicillin and 15 mg of hygromycin. pH: 5.8.

2.10.2.9. CEM-C100 agar for generation of shoots

CEM agar was prepared from (per L) MS agar with addition of 100 mg of carbenicillin and 1 mg of NAA (Duchema Biochemie). pH: 5.8.

2.10.3.10. CBM-C50-H10 agar for screening of transgenic lines

CBM-C50-H10 agar also known as media for rooting test used to screen for transgenic lines. It was prepared from CBM agar with addition of 50 mg/L of carbenicillin and 10 mg/L of hygromycin.

2.11. General methods

2.11.1. Quantification of DNA and RNA

Quantifying DNA and RNA was done using either a spectrophotometer UV-Vis (Thermo Electron Microscopy) or a NanoVue Plus (GE Healthcare). Using Spectrophotometer UV/Vis, DNA/RNA sample was diluted down to 200-fold by adding 5 µL of DNA/RNA sample to 995 µL of Milli-Q water and measuring its absorbance at 260 nm and 280 nm. Concentration of DNA and RNA (ng μL^{-1}) was calculated manually using the following formula:

DNA (ng μL^{-1}) = A260 x 50 x dilution factor
RNA (ng L^{-1}) = A260 x 40 x dilution factor

The purity of DNA/RNA was determined by calculating the ratio of the absorbance at 260 nm and 280 nm (A260/A280). For DNA, the ratio value should be 1.65-1.85 while for RNA, the ratio value should be 1.8-2.0 (University)

Another method for quantifying DNA and RNA concentration was using NanoVue Plus also known as Nanodrop. 2 µL of DNA/RNA sample was directly run on the NanoVue machine which measures concentration and absorbance of the sample at 260 nm and 280 nm.

2.11.2. Removal of genomic DNA contamination from isolated RNA

Contaminant DNA was removed using Ambion Turbo™ DNase enzyme (ThermoScientific). 50 µL sample solution containing 20 µL (50 ng) of isolated sample RNA, 5µL of Turbo™ DNase buffer, 1 µL of Ambion turbo™ DNase enzyme (2 U) and 24µL of autoclaved Milli-Q water was incubated at 37 °C for 30 min in a thermal cycler (PTC-200) machine. There were two methods to obtain the purified RNA. First: an equal volume of chloroform was added to the sample (50 µL), mixed by vortexing, and spun down at 11,337 g for 2 min. Second: 5 µL of DNase inactivation reagent was added to the mixture, incubated at RT for 10 min, mixed occasionally and centrifuged at 13,000 rpm, RT for 10 min. The upper layer (purified RNA) was pipetted out, transferred into a new tube. The purified RNA was confirmed by PCR to double check the presence of DNA in RNA sample.

2.11.3. Polymerase Chain Reaction (PCR)

PCR was used for various purposes such as PCR detection, RT-PCR, PCR genotyping, producing *att*B PCR fragment, and colony PCR. Two types of DNA polymerase enzyme were used; Taq DNA polymerase and Q5-High Fidelity (HF) DNA polymerase. However, each has a different composition of mixture reaction and reaction set up as provided by the manufacturer (NEB). PCR preparation and reaction set up used in the gene constructs creation are available in Appendix 2.

2.11.4. Agarose gel electrophoresis

DNA, RNA and PCR product were analysed on agarose gel electrophoresis and visualised under the UV transilluminator. 1.5% (w/v) agarose gel was

prepared by dissolving 1.5 g in 100 mL of 1x TAE buffer (40 µM Tris-Acetate and 1 µM EDTA) with the addition of 0.3 ng μL^{-1} of ethidium bromide. About 5 µL of samples were mixed with 1 µL of 5x loading dye contained 40% (w/v) of sucrose and 0.25% (w/v) of bromophenol blue, loaded on the gel and run at 80 volt for 30-40 min. The gel percentage for electrophoresis depends on DNA size and application. For gel DNA purification, the sample was run on lower percentage of agarose gel and at lower voltage to obtain better separation of DNA.

2.11.5. Plasmid constructions

2.11.5.1. A-Tailing with Taq DNA polymerase

PCR products (*att*B PCR product) generated using Taq DNA polymerase has blunt-ended. In order to clone the PCR products into the vector via TA cloning, it was modified by creating A-overhang using A-tailing protocol with Taq DNA polymerase (NEB). The reaction was performed in a 50 µL reaction system containing 5 µL of 10x *Taq* standard buffer, 1 µL of 10 mM dNTPs, 0.2 µL of Taq DNA polymerase (5 U/µL), 30 µL (240 ng) of blunt-ended PCR product and nuclease-free water up to 50 µL. The mixture was incubated at 72 °C for 20 min and the product (A-overhangs PCR products) further called insert was ligated into the T-vector cloning; pGEM®-T Easy vector system I using TA cloning method.

2.11.5.2. TA cloning to put an insert into pGEM®T-Easy vector

Prior to ligation, the optimum amount of insert (A-overhangs *att*B PCR product) has to be determined first and this can be done using formula below:

$$\text{ng of insert} = \frac{\text{ng of vector x size of insert (kb)}}{\text{Size of vector (kb)}} \text{ x ratio of vector:insert}$$

TA cloning was carried out by adding 3 µL (24 ng) of insert into 10 µL reaction system containing 5 µL of 2x Rapid ligation buffer (Promega), 1 µL (50 ng) of pGEM®T-Easy vector system I and 1 µL of T4 DNA ligase. The mixture was incubated at 4 °C overnight or 15 °C for 4-18 h and was used to transform NEB 10-beta competent *E. coli*.

2.11.5.3. Screening of blue/white colonies

The selection of transformants of *E. coli*::pGEM®T-Easy vector was done by spreading 100 µL of the transformation mixture over the surface of indicator LB plates supplemented with ampicillin 50 ng/µL, 0.5 mM of IPTG (isopropylthio-β-galactoside) and 80 µg/mL of X-Gal.

2.11.5.4. Restriction digestion

Restriction digest was conducted following the manufacturer's guideline (NEB/Promega). The reaction was performed in a standard 50 µL restriction enzyme digestion containing 5 µL of 10x reaction buffer, 5 µL of 10x BSA, 1 µL (10 U) of RE, DNA sample (500 ng-1000 ng) and double distilled water (ddH_2O) or Milli-Q water up to 50 µL. The restriction digestion was done at 37 °C for 3-4 h but normally overnight and the reaction was terminated by heating it up to 65 °C for 20 min.

2.11.5.5. Purification of digested product

5 µL of the digested product was analysed on an agarose gel and the rest of the volume was purified using a PCR purification kit (QIAGEN), before using it in the ligation process. About 45 µL of digested product was added to 5 volume of buffer PB (225 µL) and mixed by pipetting. The solution was transferred into a spin column, added with 10 µL of 3M sodium acetate (pH. 5) to bind the DNA and was centrifuged at 11,337 g and RT for 1 min. The pellet was washed using 750 µL of buffer PE, centrifuged at 11,337 g and RT for 1 min and the pellet (DNA) was then eluted in 30 µL of sterile Milli-Q water.

2.11.5.6. Ligation using Promega T4 DNA ligase

The ligation was performed in a 0.2 mL PCR tube with total working volume of 10 µL. The reaction mixture containing 5 µL (50 ng) of the vector, 1 µL of 10x rapid ligation buffer, 3 µL (3 ng) of the insert, 1 µL (10 U) of T4 DNA ligase and nuclease-free water up to 10 µL was incubated at 4 °C overnight or 15 °C for 4-18h. 1 µL of the product was then used to transform competent *E. coli* cells.

2.11.5.7. The GATEWAY Cloning Technology

The Gateway cloning technology involves two (2) steps; creating an entry clone via BP recombination reaction and an expression clone via LR recombination reaction. The gateway cloning was performed following the manual from the manufacturer (Life Technologies).

BP recombination reaction

In this study, BP reaction, which is catalysed by BP Clonase™ enzyme mix, is the recombination reaction between *att*B-PCR product or a linearized *att*B-pGEM or pKannibal vectors with an *att*P-pDONR/Zeo to create an *att*L-entry clone (Figure 2.3).

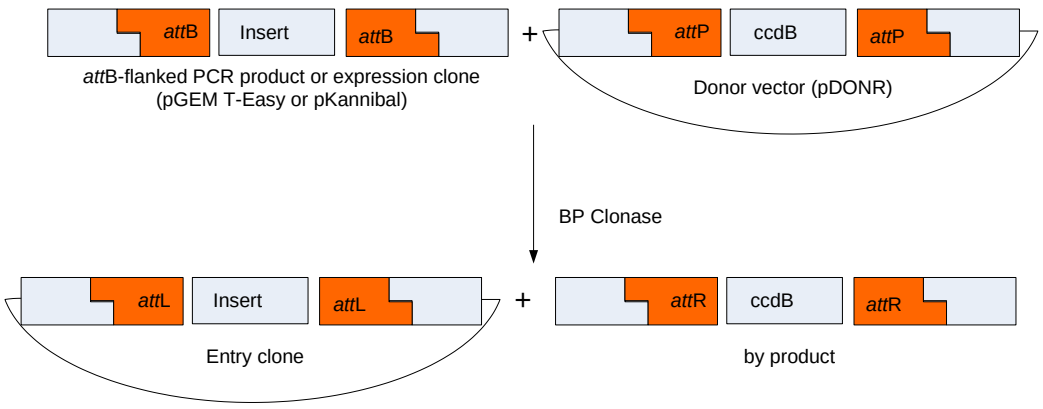


Figure 2.3. BP recombination reaction in the GATEWAY cloning technology.

https://tools.thermofisher.com/content/sfs/manuals/gatewayman.pdf with some modifications.

The BP reaction was performed in a 10 µL standard reaction consisting of 15-150 ng of linearised *att*B expression clone (pGEM or pKANNIBAL) or *att*B-flanked PCR fragment, 1 µL (150 ng) of pDONR/Zeo harbouring the Zeocin resistance gene, TE buffer pH 8.0 up to 8 µL and 2 µL of BP clonase™ II enzyme mix. The reaction mixture was incubated at 25 °C overnight and was terminated by adding 1 L of proteinase K. It was then incubated at 37 °C for 10 min and about 1 µL of BP product was used to transform competent *E. coli*. The transformants were grown on low salt LB agar medium contained Zeocin 50 µg mL^{-1}

LR recombination reaction

The LR reaction, which is catalyzed by the LR Clonase™ enzyme mix, which mediates recombination of an *att*L-entry clone (figure 2.3) with an *att*R-pCAMBIA 1305.1 as the destination vector to create an *att*B-expression clone (Figure 2.4).

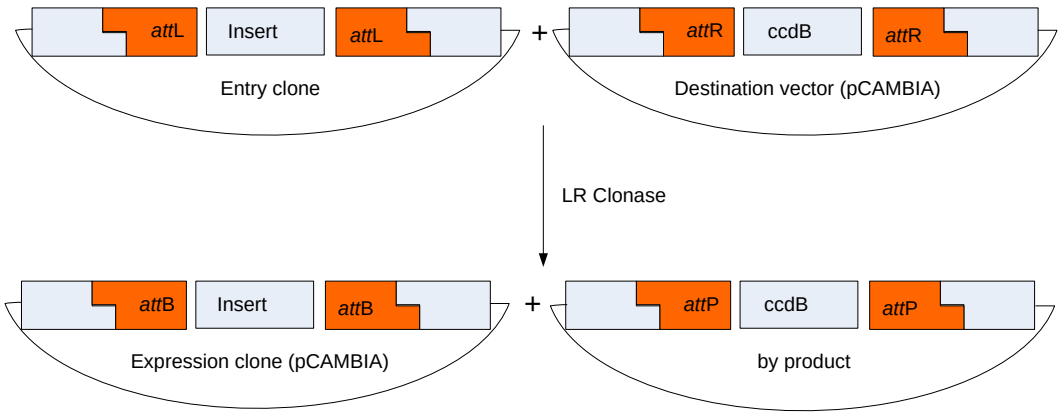


Figure 2.4. LR recombination reaction in the GATEWAY cloning technology.

https://tools.thermofisher.com/content/sfs/manuals/gatewayman.pdf with some modifications.

The LR reaction was performed in a 10 µL reaction containing 50-150 ng of an entry clone, 1 µL (150 ng) of destination vector (pCAMBIA 1305.1), TE buffer pH 8.0 up to 8 µL and 2 µL of LR clonase™ II enzyme mix. The reaction mixture was incubated at 25 °C overnight and was terminated by

adding 1 L of proteinase K. It was then incubated at 37 °C for 10 min and about 1 µL of LR product was used to transform competent *E. coli*. The transformants were grown on LB agar medium with Kanamycin 50 µg mL^{-1}.

2.11.6. Transformation of competent *E. coli* cells

Following protocol from manufacturer (NEB), about 1-5 µL (1-100) ng of plasmid DNA was added into 50 µL of NEB 10-beta competent *E. coli* and mixed gently by flicking the tube for several times. The mixture was incubated on ice for 30 min, heated up at 42 °C for 30 sec. and incubated on ice for 5 min. 950 µL of S.O.C liquid medium was added into the mixture, incubated at 37 °C for 60 min. About 100 µL of the transformed cells was spread on LB agar contained an appropriate antibiotic and the plate was incubated at 37 °C overnight. The colonies were counted and the transformation efficiency was calculated using the following formula:

$$\text{TE (CFU/µg)} = \frac{\text{number of colony (CFU)}}{\text{(µg of DNA x dilution factor)}}$$

2.11.7. Preparation of Electro-competent Agrobacterium cells

A single colony of either *A. tumefaciens* strain LBA4404 or GV 3101 from a 50% glycerol stock culture at -70 ºC initially grown on LB agar was inoculated into 5 mL of LB broth supplemented with appropriate antibiotics and incubated at 28 ºC, 200 rpm for 1-2 days. The culture was scaled up to 100 mL in a 250 mL flask, incubated at 28 ºC, 200 rpm and its cell density was checked periodically until the OD_{600} value reached about 1. Once the OD reached the expected value, 50 mL of culture was centrifuged using benchtop centrifuge (Hettich Universal 32R) at 4 x 100 g, 4 ºC for 15 min. The pellet was washed 2x with 1 volume and 0.5 volume of chilled-milliQ water, respectively, and centrifuged at 4 x 100 g, 4 ºC for 15 min. The pellet was then washed with 1 mL of chilled-10% glycerol, vortexed and

centrifuged at 4 x 100 g, 4 ºC for 15 min. In the final step, the pellet was dissolved in 300 µL of chilled 10% glycerol, mixed with a pipette, and aliquots of 50 µL in a 1.5 mL tube were stored at -70 ºC until use.

2.11.8. Agrobacterium transformation

Agrobacterium electrotransformation was performed using the Bio-Rad Gene Pulser system (Bio-Rad, Richmond, CA, U.S.A.) according to the manufacturer's manual instructions. 1 µL of 15 ng/L expression vector containing the gene target (Figure 2.4) and 40 µL of competent Agrobacterium cells were mixed in a cuvette (Biorad) that had been pre-chilled (-20 ºC) for 20 min. The mixture placed in the Bio-Rad Gene Pulser to transform the cells. 1 mL of LB or 2YT liquid medium was added into the cuvette without antibiotics and incubated at 28 ºC, 200 rpm for 3 h. The cultures were spread on LB plate selection medium contained appropriate antibiotics and were incubated at 28 ºC for 2-3 days until colonies appeared.

2.11.9. Isolation of bacterial plasmid DNA

Bacterial plasmid DNA was isolated from 4-5 mL overnight-culture using the QIAprep Spin Miniprep kits (QIAGEN). The culture was centrifuged at 11,337 g, RT for 10 min. The pellet was re-suspended using 250 µL of buffer P1, mixed with 250 µL of lysis buffer P2 and 350 µL of buffer N3 was added. The solution was gently mixed by inverting the tube several times. The mixture was then centrifuged at 11,337 g, RT for 10 min and the supernatant was placed into a spin column and centrifuged at 11,337 g, RT for 1 min. 750 µL of buffer PE was added to the spin column containing the pellet and spun down at 13,000 rpm, RT for 1 min. To remove buffer PE completely, it was centrifuged again at 11,337 g, RT for 1 min. In the last step, the plasmid DNA was eluted with 50 µL of milliQ water, centrifuged at 11,337 g, RT for 1 min. and was stored at -20°C until used.

In order to isolate plasmid DNA in a larger volume, the QIAGEN® Plasmid

Midi kit was used. 50 mL of overnight-cultured was centrifuged at 4,000 rpm, 4°C for 15 min. The pellet was re-suspended in 6 mL of buffer P1 followed by the addition of buffer P2 and incubation at RT for 5 min. Buffer P3 was added and the mixture was incubated on ice for 15 min before it was centrifuged at 11,337 g, 4°C for 30 min. The supernatant was applied into a QIAGEN tip 100 that had previously been equilibrated using 4 mL buffer QBT. The supernatant was allowed to flow through the QIAGEN tip by gravity. The QIAGEN tip was then washed twice (2x) using buffer QC and the DNA was eluted using 6 mL of buffer QF. The eluted DNA was precipitated using 4.2 mL of isopropanol and centrifuged at 11,337 x g, 4°C for 30 min. The pellet was washed using 2 mL of ethanol and then centrifuged at 11,337 g, 4°C for 10 min. The plasmid DNA was dissolved in 80 µL of nuclease-free water after air-drying for 30 min.

2.11.10. DNA sequencing and analysis

Two types of samples were used for sequencing in this study: purified PCR product and bacterial plasmid DNA. About 70-100 ng/µL of samples were sent to Eurofins Company along with specific primers for sequencing (see 2.5.9) following the guidance of sample submission from the company (https://www.eurofinsgenomics.eu/). The results were then analysed using Geneious software (Kearse et al., 2012).

2.12. Arabidopsis Research Methods

2.12.1. Identification of Arabidopsis T-DNA insertion mutants

Arabidopsis mutants were used to study the function of selected cassava genes. The mutants were identified by BLASTing gene of interest sequences: At4g34131, for Scopoletin-glucosyltransferase, and At3g09260 for Scopolin-beta-glucosidase on Arabidopsis gene mapping tool website (http://signal.salk.edu/cgi-bin/tdnaexpress) from Salk Institute Genomic Analysis Laboratory (SIGnAL).

2.12.2. Surface sterilisation of Arabidopsis seeds

Arabidopsis seeds in a 1.5 mL tube were immersed in 800 µL of 70% (v/v) ethanol, mixed by inverting the tube for 5 min and the supernatant was discarded. About 800 µL of a mixture containing 50% (v/v) bleach and 0.1% (v/v) tween 20 was added, and mixed for 5 min, then thoroughly rinsed with sterile milliQ water for 4-5 times until the bleach was completely removed. In the last step, 800 µL of 0.15% (w/v) agar was added and the seeds were stratified at 4 ºC for 2-3 days.

2.12.3. Growth of Arabidopsis on agar and soil medium

Arabidopsis seeds were grown on agar for roots analysis. After 2-3 days stratification, the seeds were planted on MS agar (see 2.9.1) in a 100 mm square plates (Fisher) as illustrated in figure 2.5.

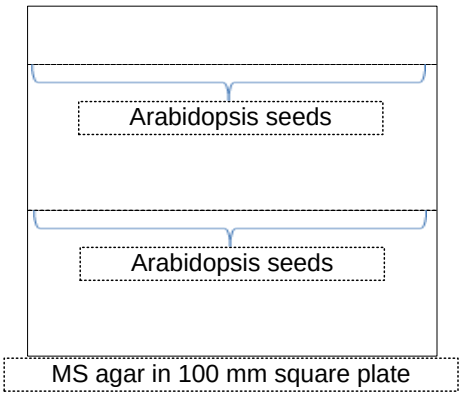


Figure 2.5. Planting method of Arabidopsis seeds on an agar plate. The plates were placed vertically in the growth room under controlled conditions at 21-22 ºC, 40-60% relative humidity and 16 h daylight. The roots were collected after 4-5 weeks for biochemical analysis.

Arabidopsis seeds were grown on soil for several purposes such as seed collection, DNA extraction, genotyping and transformation. After 2-3 days of stratification, the seeds were planted on soil medium (see 2.9.2) in a tray as shown in figure 2.6. The trays were kept in the growth room under controlled condition at 21-22 ºC, 40-60% relative humidity and 16 h daylight.

Figure 2.6. Planting method of Arabidopsis seeds on soil medium in the growth room under controlled conditions at 21-22 ºC, 40-60% relative humidity and 16 h daylight.

2.12.4. Analysis of homozygous knock-out lines

This analysis consists of two steps; isolation of genomic DNA from leaves and PCR genotyping using specific T-DNA primers.

2.12.4.1. Isolation of DNA from Arabidopsis leaves

Arabidopsis genomic DNA was isolated from 3-4 week-old leaves using a modified CTAB method adopted from Alonso-Stepanova (2014). A piece of rosette leaf (about 15-20 mg) was placed in a 1.5 mL tube containing 200 µL of extraction buffer (0.14 M of d-sorbitol, 0.22 M of Tris-HCl pH 8, 0.022 M EDTA pH 8, 0.8 M NaCl, 0.8% (w/v) CTAB and 0.1% (w/v) N-lauroyl sarcosine) and homogenised using a pillar drill machine with a plastic rod. The homogenate was incubated at 65 °C for 5 min, 100 µL of chloroform was added and mixed by vortexing for 30-60 sec before being centrifuged at 11,337 g, room temperature (RT), for 5 min. The supernatant (upper phase) was transferred into the new 1.5 mL tube and 150 µL of isopropanol was added. It was then incubated at RT for 15 min and was centrifuged at 11,337 g, RT for 20 min. The supernatant was discarded and 500 µL of 70% (v/v) ethanol was added to the pellet. The mixture was mixed by inverting

the tube and was spun down. It was then air-dried for 20-30 min until ethanol has been completely evaporated then the pellet was re-suspended using 50 µL of Milli-Q water. The isolated DNA was stored at -20 °C until use.

2.12.4.2. T-DNA insertion lines genotyping

Arabidopsis T-DNA insertion lines genotyping was carried out using PCR with T-DNA specific primers (see 2.5.5) and PCR condition described in the PCR section (Appendix II).

2.12.5. Isolation of total RNA from Arabidopsis leaves

Arabidopsis total RNA was isolated for gene expression analysis in selected homozygous lines. It was isolated from leaf samples using “Spin & Vacuum” (SV) total RNA isolation system (Promega) protocol. Isolated total RNA was then purified before it was used to create cDNA.

2.12.6. Synthesis of cDNA and analysis of gene expression (RT-PCR)

Synthesis of cDNA was done using RevertAid First-Strand cDNA synthesis kit (Thermo Scientific) which was performed with a 20 µL system. 12 µL of mixture solution contained 5 µL (240 ng) of purified RNA, 5 µL of nuclease-free water, 1 µL of 100 µM oligo (dT)18 primer and 1 µL of 100 µM random hexamer primer was incubated at 65 °C for 5 min. It was then incubated on ice for 5 min. 4 µL of 5x reaction buffer, 1 µL of RiboLock RNase inhibitor (20 U/µL), 2 µL of 10 mM dNTP mix and 1 µL of RevertAid M-MuLV reverse transcriptase (20U/µL) were added to the 12 µL sample solution, gently mixed and spun down. The next step was to incubate the mixture at 25 °C for 5 min, 42 °C for 60 min and 70 °C for 5 min, respectively. The synthesised cDNA then used for gene expression analysis or stored at -80 °C until use.

Analysis of gene expression in homozygous knockout lines was done using

gene specific primers (see 2.5.8) with PCR conditions shown in Appendix II. It was done to confirm that the corresponding gene in Arabidopsis mutant has been knocked out before using it in plant transformation.

2.12.7. Creation of overexpression constructs for functional studies in Arabidopsis

Full-length cDNA of gene of interest was amplified by PCR with gene specific primers and was cloned into TA vector, pGEM® T-Easy vector system I. This vector was used in the Gateway Cloning Technology (Life Technologies) with several steps mentioned in section of plasmid constructions (See 2.11.5) to create expression constructs. Four overexpression constructs under the control of two promoters; constitutive CaMV35S and root-specific *St*PATATIN promoters were created.

2.12.8. Transformation of Arabidopsis knockout lines

2.12.8.1. Preparation of bacterial culture

A. tumefaciens strain GV 3101 was used for Arabidopsis transformation. The initial culture was prepared from a 50% (v/v) glycerol stock stored at -70 ºC, by streaking the stock cultures on LB agar selection medium (see 2.9.5.2). The agar plate was incubated at 37 ºC for 2-3 days until single colonies appeared. A single colony was inoculated into 5-mL LB liquid medium (see 2.9.5.1). The culture was then incubated at 37 ºC, 200 rpm overnight, until OD_{600} value between 0.7-1.0. The overnight-culture was centrifuged to obtain the pellet, which was used for plant transformation.

2.12.8.2. Preparation of solution buffer for transformation

Solution buffer for Arabidopsis transformation was prepared as mentioned in 2.9.5.3. The pellet was dissolved using this buffer with the OD_{600} adjusted to 0.7. A surfactant, Silwet-L 77 (Pam's green Laboratory, Delaware Biotechnology Institute) of 0.01% (v/v) was added before transformation.

2.12.8.3. Arabidopsis transformation

Arabidopsis transformation was done using dip-floral method (Clough and Bent, 1998). About 5-week-old Arabidopsis knock out lines were used for transformation. A day before transformation, opened siliques were cut off the plant and remained flowers only. The flowers were dipped in the bacterial suspension (2.12.8.2) for 10 second, 2 times. The transformed plants were let dry for few hours and were covered with black plastic overnight. The plastic was opened on the following day and the transformed plants grown until mature to produce seeds, which took another 3-4 weeks. Mature seeds (F_0) were harvested and were stored in a 1.5 mL micro centrifuge tube at room temperature or used for further analysis.

2.12.9. Selection of transgenic Arabidopsis

Seeds from transformed plant (F_0) were screened for transgenic lines. 2 mg of seeds, equal to about 2000 seeds, were spread on LB agar selection medium containing the plant selection antibiotic, hygromycin (see 2.9.5.4). The plates were wrapped in aluminium foil and kept in the growth room (21-22 ºC) for 5 days. Seedlings that developed long hypocotyls were transgenic and then transferred gently to soil medium (see 2.9.2). Seeds from selected transgenic lines (F1) were used directly for biochemical analysis.

2.12.10. Analysis of scopoletin and scopolin in wild type and transgenic Arabidopsis

2.12.10.1. Preparation of Arabidopsis roots sample

Seeds (F1) were grown on square plates containing MS agar (see 2.10.3.). About 4-5-week-old seedlings were harvested for the roots. The roots sample was oven-dried at 50 ºC for 2-3 hours or until completely dry. It was then stored at -70 ºC or directly used for biochemical analysis.

2.12.10.2. Extraction of Scopoletin and Scopolin from Arabidopsis roots

10-15 mg of Arabidopsis root powder in a 5 mL screw-cap glass vial (Fisher Scientific) was used. 2.0 mL of methanol (MeOH)-HPLC grade containing 200 ng/mL of the first internal control, 4-Methyl Umbelliferone (4-MU) was added and was incubated on a shaker (IKA-VIBRAX-VXR) at room temperature, 100-150 rpm and overnight or for 16 hours. The extract/liquid phase was filtered through a Nalgene syringe filter 0.45 µm PES (Thermo Scientific) into a 2 mL screw-cap microfuge tube (Molecular BioProducts). In order to evaporate the extraction solvent, the filtrate was then vacuum-concentrated in a Savant SpeedVac vacuum concentrator (Thermo Fisher) for about 3-4 h or until the solvent was completely evaporated. Dried extract was then reconstituted with 200 µL of MeOH-HPLC grade containing 1 µg m/L scoparone as the second internal control and was stored at 4℃ for a few hours or overnight to re-dissolve the extract. The re-dissolved extract was vortexed vigorously and centrifuged at 11,337 g for 5 min. to spin down any unwanted particles. 150 µL of the extract was transferred to a 300 µL screw-cap vial (Waters) and sent to Department of Pharmacy and Pharmacology, University of Bath for biochemical analysis using LC-MS. Internal controls; 4-MU and scoparone, which are structurally similar to scopoletin, were used as a correction factor of the analysis.

Details of LC-MS method as follows:

10 µL of sample was injected into a liquid chromatography machine; Acquity UPLC BEH C18, 1.7 µM, 2.1 x 50 mm reverse phase column (Waters, Milford, MA, USA), flow rate of 0.3 mL min^{-1}, two mobile phases A and B were used, consisting of 0.1% (v/v) formic acid in water, and 0.1% (v/v) formic acid in methanol, respectively. Mass Spectrophotometry was conducted using a MaXis HD quadrupole electrospray time-of-flight (ESI-QTOF) mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany), operated in ESI positive-ion mode. Scopoletin, scopolin, esculetin and

esculin were identified with a mass-to-charge (*m/z*) ratio of 355.1024, 341.0867, 193.0495, 179.0339 and 163.0390 within 0.005 Da, respectively. Data analysis was performed using Data Analysis software version 4.3 (Bruker Daltonik GmbH, Bremen, Germany).

2.13. Cassava Research Methods

2.13.1. Identification of homologous cassava genes

Homologous cassava genes for scopoletin-GT and scopolin-BG were identified by BLASTing the predicted amino acid sequence of the reference genes on the Plant Genomic Resource, Phytozome database (Goodstein et al., 2012).

2.13.2. Isolation of genomic DNA from cassava leaves

Cassava genomic DNA was isolated from leaves using modified a CTAB (Cetyltrimethyl ammonium bromide) method adopted from (Huang et al., 2013). Approximately 100 mg of young cassava leaves were placed in a 1.5 mL tube contained glass beads and 2% (w/v) PVPP, dipped in liquid nitrogen (N_2) to freeze the sample and ground to a fine powder using a triturator silamat S5 machine for 10 sec.

1 mL DNA^{simp} lysing buffer (2 M NaCl, 25 M EDTA, 200 mM Tris, 2% (w/v) CTAB, 2% (w/v) PVPP, 1% (w/v) LSS and 20 mM Borax) was added into the tube, incubated at 65 °C for 30 min, centrifuged at 11,337 g, room temperature (RT) for 10 min. About 500 µL of supernatant was transferred into the new 1.5 mL tube. 500 µL of 70% (v/v) Isopropanol was added, gently mixed by inverting the tube, incubated at -20 ºC for 30 min and centrifuged at 11,337 g, RT for 5 min. 1 mL of pre-chilled (4 ºC) of 75% (v/v) ethanol was added into the pellet, vortexed and centrifuged at 11,337 g, RT for 2 min. 1 mL of 100% (v/v) ethanol was added to the pellet and centrifuged at 11,337 g, RT for 2 min. The pellet was air-dried for 15-20 min, eluted in 50 µL of DW, incubated at 65 ºC for 10-15 min, vortexed and stored at -20 ºC

until use.

2.13.3. Isolation of RNA from cassava roots

Total RNA from cassava was isolated using a modified CTAB (Cetyltrimethyl ammonium bromide) method adapted from (Chang et al., 1993).

Day 1. About 100 mg of cassava roots was ground to fine powder with liquid nitrogen (N_2) in a sterile pestle and mortar (baked overnight at 180 ºC). The ground sample (fine powder) was immediately added to pre-warmed (65 °C, 10 min) extraction buffer (2M NaCl, 25 mM EDTA, 100 mM Tris-HCl, 2% (w/v) PVP) contained 2% (v/v) mercapto-ethanol which had been freshly added. The solution was mixed by inverting the tubes and was incubated at 65 °C for 15 min with vigorous shaking every 3 min. 500 µL of chloroform:IAA (24:1) was added, mixed well by vortexing for 10-15 sec., centrifuged at 11,337 g, room temperature (RT) for 10 min. and 400 µL of the supernatant was transferred into a new 1.5-mL tube. 400 µL of chloroform:IAA (24:1) was added, mixed by vortexing for 10-15 sec., and centrifuged at 11,337 g, RT for 10 min. About 300 µL of the upper phase was transferred into a new 1.5-mL tube and 300 µL of chilled isopropanol and 150 µL of 10 M LiCl (2 M final concentration) were added, mixed well and incubated at 4 °C overnight.

Day 2. The overnight-incubated sample was centrifuged at 11,337 x g, RT for 30 min. The pellet was re-suspended with 1 mL of Sigma water, 250 µL of 10 M LiCl was added, incubated on ice for 3 h and centrifuged at 11,337 g, RT, for 10 min. The pellet was re-suspended in 250 µL of Sigma water (DNA and RNA-free nuclease water), 25 µL of 3M Sodium Acetate, and 1 mL of absolute ethanol were added, and then the mixture was incubated at -20 °C for 3 h, followed by centrifugation at 11,337 g, RT for 30 min. The pellet was then dissolved in 50 µL of RNase-free water (sigma water) and

stored at -80 °C until use.

2.13.4. Synthesis of cDNA and analysis of RT-PCR

This was done using the same kit and protocol from ThermoFisher (see 2.10.6).

2.13.5. Creation of RNAi and Overexpression constructs for cassava transformation

To create RNAi constructs, pKANNIBAL (CSIRO, Australia) were first used to generate hairpin constructs expressing sense and antisense of gene of interest fragment. The pKANNIBAL containing hairpin constructs were cloned into pGEM T-Easy vector system I via TA cloning and used it in the GATEWAY cloning technology. While for overexpression constructs creation, it was created using the same method as mentioned in section 2.11.7. The gene constructs were created under the control of two promoters; constitutive Cauliflower Mosaic Virus (CaMV) 35S and root-specific *StPATATIN* promoters. Overexpression construct harbouring GUS Plus gene (reporter gene) was used as a control of the transformation.

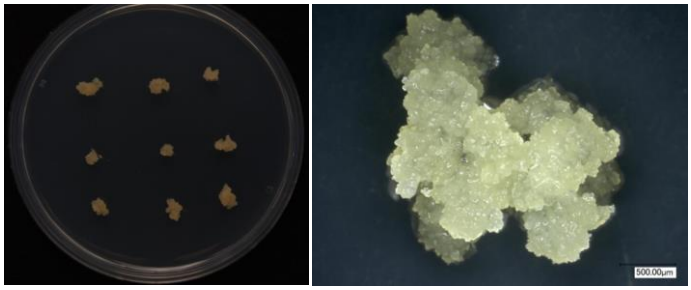
2.13.6. Transformation of Cassava Friable Embryogenic Callus (FEC)

Transformation of cassava FECs was carried out at the Plant Biotechnology Laboratory, ETH-Zurich, Switzerland following method that of (Bull et al., 2009) with several modifications. The transformed FECs were brought to University of Bath for maturation of transformed FECs, regeneration and analysis of putative transgenic lines.

2.13.6.1. Preparation of Cassava FEC

FEC was initially prepared by Dr. Ima Zainuddin, ETH-Zurich. It was induced from axillary buds, maintained on GD agar medium and transferred every 4 weeks onto fresh GD agar medium containing 9 clumps in one plate (figure 2.7a). Two-week-old FEC were used for transformation (Figure 2.7b), one

gene construct normally requiring 6 plates of FEC for replication.



a b

Figure 2.7. Material FECs for cassava transformation. a) FEC on GD agar medium under digital microscope camera (Canon EOS 1100D, f/6.3, 1/50 sec, ISO-400), b) FEC on GD agar medium under digital microscope VHX-1000 series (Keyence).

2.13.6.2. Preparation of *A. tumefaciens* strain LBA4404 culture

A. tumefaciens strain LBA4404 was used for cassava transformation. The initial culture was prepared from 50% glycerol stock that was stored at -70 ºC by streaking on 2YT or LB agar selection medium (see 2.10.2.2). The agar plate was incubated at 37 ºC 2-3 days until colonies appeared. A single colony was inoculated into 5-mL LB liquid medium (see 2.10.2.3). The culture was then incubated at 37 ºC, 200 rpm, overnight until an OD_{600} value between 0.7-1.0 was obtained. The overnight-culture was centrifuged using a bench-top centrifuge at 5000 rpm, RT for 10 min. The pellet was re-suspended with GDS and the OD600 value was adjusted to 0.5. 200 µM acetosyringone was added to the bacterial suspension, which was placed on a shaker at 10-20 rpm for 15 min. The culture was then ready for plant transformation.

2.13.6.3. Inoculation and co-cultivation

About 2 drops of the Agrobacterium suspension were dropped on to each clump of two-week-old FECs, these and were co-cultivated at 25-28 ºC, 16 h light for 4 days. After co-cultivation, 3 plates of transformed FECs were collected and added into a 50-mL conical tube (Greiner) containing 30 mL

of GDS supplemented with 500 mg/L carbenicillin for washing step in order to remove the Agrobacterium. The culture suspension was vortexed for 10 sec. and allowed FECs settle down. The supernatant was discard and the washing step was repeated 4-5 times until the solution was transparent/clear indicating most of Agrobacterium had been removed. In the last washing, about 5 mL of FECs suspension was kept then poured onto a sterile 100 μm nylon mesh. The FECs-containing mesh was transferred to sterile filter paper to absorb/remove any excess GDS. The mesh was then placed on GD-C250 agar and was incubated at 25-28ºC, 16 h of light for 4 days for the transformed FECs recovery.

2.13.6.4. Maturation and development of transformed FECs

These steps were done by transferring transformed FECs to fresh selection medium every week. Using nylon mesh facilitates the transfer step without disrupting the FECs and permits close regulation of media components such as pH, salt, vitamin and antibiotic in the development of FECs.

After 4 days' incubation above, the mesh containing the transformed FECs was transferred to fresh GD-C250 agar plate supplemented with plant selection antibiotic, Hygromycin 5 mg/L (GD-C250-H5) and was incubated for one week. After one-week incubation, it was transferred to fresh GD-C250 agar plate with higher concentration of Hygromycin of 8 mg/L ((GD-C250-H5). After one-week incubation, it was transferred to fresh GD-C250 with 15 mg/L Hygromycin (GD-C250-H15). All incubation was done at 25-28 ºC and 16 h of light.

2.13.6.5. Regeneration of putative transgenic line

After one-week incubation on GD-C250-H15, the transformed FECs were transferred on MSN-C250-H15 agar and incubated at 25-28 ºC, 16 h of light for 10 days. The transformed FEC were repeatedly transferred to MSN-C250-H15 agar until small, greenish cotyledon appeared which normally

takes 6-7 transfers. The expanded green cotyledons were then transferred onto CEM-C100 agar medium and incubated for 1-2 weeks or until cotyledons developed stem and leaves. The developing cotyledons were then cultured in CBM agar medium for growth until fully developed as a plantlet.

2.13.7. Selection and analysis of transformed FECs and transgenic cassava lines

2.13.7.1. Test of transformation success by GUS assay

FECs or leaf samples were added to 1 mL of GUS staining buffer containing 890 µL of 10 mM Tris (pH 7.2), 50 mM NaCl, 100 µL of X-Gluc and 10 µL of 10% (v/v) Triton X-100. It was incubated at 37 ºC overnight and photographed. The control should turn blue as it carries the GUS Plus reporter gene that will generate a blue complex by reacting with X-Gluc. Samples were washed with 70% (v/v) ethanol and were stored at room temperature.

2.13.7.2. Preliminary selection of transgenic lines using rooting test

The plantlets were screened to identify putative transgenic lines by the rooting test which was performed in a 50-mL conical tubes. Shoots of 4-week-old plantlets were sub-cultured in CBM agar medium supplemented with 10 mg/L Hygromycin. After 2 weeks, plantlets that produced roots under hygromycin selection medium were considered as probable transgenic lines. Moreover, the presence of the insert was confirmed by PCR.

2.13.7.3. Confirmation of insert by PCR

The insert in putative transgenic lines was confirmed by PCR using genomic DNA from leaves and gene specific primers (see 2.5.10) with the reaction conditions shown in Appendix II.

2.13.7.4. Analysis of insert copy number by Southern blot

***A. Preparation of DIG-labelled Hygromycin probe*.** 50 μL of Hygromycin-probe (Table 2.6) was prepared in a 0.2 mL tube and run on PCR (Peltier Thermal Cycler PTC-200) with the following reaction condition: 95 ºC for 2 min, 95 ºC for 30 sec., 58 ºC for 30 sec., 72 ºC for 1 min,72 ºC for 5 min and 12 ºC forever (holding time) with 30 cycles. The PCR product was run on 1.2% (w/v) agarose gel electrophoresis and purified using QIAquick Gel Extraction Kit (Qiagen) following protocol from manufacturer. The gel-purified probe was eluted in 50 μL of sterile DW and was stored at -20 ºC until use.

Table 2.6. Composition of Hygromycin probe.

No	Component	Volume (μL)
1	10X Taq buffer	5
2	DIG dNTPs	5
3	Hygromycin-forward primer (10 μM)	0.5
4	Hygromycin-reverse primer (10 μM)	0.5
5	Taq DNA Polymerase (5 U/μL)	1
6	Plasmid DNA (pCAMBIA 1305.1) (15 ng/μL)	5
7	ddH_2O (Milli-Q water)	33
	Total volume	50

B. Preparation of reagents for southern blotting

All reagents composition including 20X SSC, 20% SDS, depurinating solution, denaturing solution, neutralisation solution, washing solution 1 (W1), washing solution 2 (W2), washing solution 3 (W3), washing buffer (WB), B1 solution, B2 solution, and B3 solution are available in Appendix 3.

Southern blotting-Day 1

C. Digestion reaction. 10 μg of cassava genomic DNA was digested using restriction enzyme, *Hind*III (NEB) in a 1.5 mL tube, 150 μL total volume and at 37 ºC. Plasmid DNA from pCAMBIA 1305.1 (5 pg/ μL) was used as a positive control in blotting reaction. A master mix reaction (Table 2.7) was prepared and incubated at 37 ºC for 3 h in water bath.

Table 2.7. Master mix for digestion reaction using *Hind*III.

No	Component	Volume (μL)
1	Cassava genomic DNA (500 ng/μL), plasmid DNA (pCAMBIA 1305.1) (5 pg/ μL)	20
2	10X buffer NEB 2	15
3	10X BSA	5
4	*Hind*III (20 U/μL)	5
5	DNase, RNase, Protease, free water (Sigma)	105
	Total Volume	150

D. Ethanol Precipitation. After 3 h incubation, 15 μL of 3M NaOAC (pH 4.8) and 350 μL of 95% (v/v) ethanol were added into the 150 μL mixture reaction. It was incubated at -20 ºC for 2 h, centrifuged at 13,000 rpm, RT for 15 min and the pellet was washed.

E. Washing and eluting DNA sample. 1 mL of 70% (v/v) ethanol was added to the pellet. It was vortexed for 20-30 sec and then centrifuged again at 13,000 rpm, RT for 10 min. The pellet was air-dried for 10-15 min, dissolved in 15 μL of ddH_2O (milliQ water), incubated at 65 ºC for 20 min, vortexed, spun down and run on a 0.8% (w/v) agarose gel.

F. Agarose gel electrophoresis. 5 μL of 5x loading dye was added to 15 μL of digested DNA and run at 1 V/cm overnight and in Tris-Borate-EDTA

(TBE) as running buffer. A DIG-ladder was used as a DNA size ladder.

Southern blotting-Day 2

G. DNA visualisation under UV light. The gel was stained with 0.3 mg/ml Ethidium Bromide for 15 min and was de-stained in water for 20 min. The gel was observed under UV light for DNA visualisation, documentation and was then prepared for membrane transfer.

***H. Transferring DNA to membrane*.** Firstly, the gel was cut at the top left corner to mark the gel orientation. The gel was incubated in 300 mL of depurinating buffer for 30 min with gently shaking on a shaker, washed 2x with sterile-DW, incubated in 300 mL of denaturation buffer for 30 min with gently shaking and washed 2x with sterile-DW. The gel was then incubated in 2x in neutralisation buffer for 15 min each and transferred to nylon membrane (GE Healthcare, Life Sciences) with the following reaction set up (Figure 2.8) which was performed at RT overnight.

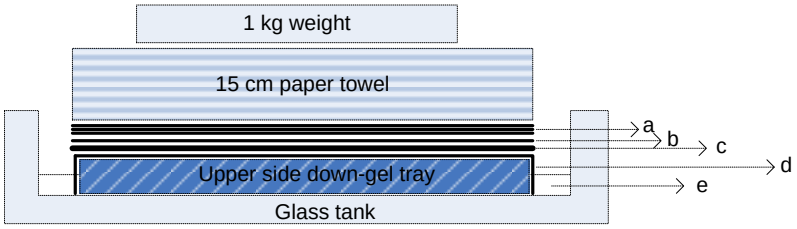


Figure 2.8. Southern blotting for copy number analysis. Overnight gel-membrane DNA transfer set up: a. 3 pieces of Whatman paper, b. nylon membrane, c. upper side down gel, d. 22 cm x 20 cm Whatman paper, e. 20x SSC buffer.

Southern blotting-Day 3

I. Hybridization with DIG-labelled probe. The membrane was cut at the top left to ensure the orientation. The membrane was dried in an oven at 80 ºC for 10-15 min and placed under the UV light at 254 nm for 2 min for UV-crosslinking. The membrane was then put in a hybridization tube that

contained 10 mL of DIG easy hybridization solution (Rohce) and pre-hybridized at 42 ºC for 4.5 h. 10 mL of fresh DIG easy hybridization solution containing 50 µL of pre-heated (95 ºC, 5 min) DIG-labelled hygromycin probe (point A) was added to the tube and incubated at 42 ºC overnight.

Southern blotting-Day 4

J. Washing membrane. After overnight hybridization, the probe was poured into a 15 mL tube and stored at -20 ºC for next use. The membrane was washed 3x with 300 mL W1 solution for 15 min each, followed by 300 mL of pre-warmed (68 ºC) W2 for 15 min and 300 mL of pre-warmed (72 ºC) of W3 solution for 30 min. The membrane was then incubated in 300 mL of WB solution for 3 min then in 160 mL of B2 solution for 1 h. About 1.5 µL of antibodies (Rohce) was added into 40 mL of B2 and was used to incubate the membrane for 30 min. The membrane was then washed 5x with WB solution (1x for 5 min, 2x for 10 min, 1x for 1 hr and 1x for 30 min). After washing, the membrane was incubated in 93 mL of B3 solution for 5 min., and then overlaid with 7 mL of B3 solution containing 40 µL of CDP star (Rohce) for 5 min., dried on Whatman paper for 5 min, sealed with Saran plastic wrap (DowBrands, USA) and put in a film cassette (Kodak BioMax light film, Sigma Aldrich). It was then incubated at 37 ºC overnight.

Southern blotting-Day 5

K. Developing film cassette. The film was developed in the dark room using OPTIMAX X-Ray Film Processor Modell 1170-1-0000 (PROTEC GmbH & Co KG, Germany).

2.13.8. Cassava plant growth in a glasshouse

2.13.8.1. Propagation of cassava *in vitro* culture

Shoots and stems from 4 to 5-week-old cassava *in vitro* cultures were cut and was transplanted in a 330 mL Polystyrene (PS) container (Greiner) containing CBM agar with 3 g/L (w/v) gelrite. Ideally, 3 explants are grown

in one container for maintenance. However, for multiplication, 5 explants were grown in one container to save propagation time and medium. The plants were cultured in the cassava growth room at the University of Bath under controlled conditions of 25-28ºC, >50% relative humidity and 16 h daylight.

2.13.8.2. Acclimatization of cassava *in vitro* culture in growth room

There are two main preparations in this step; soil medium in a tray with 24 cells (3 cm x 3 cm) and the planting material. Soil medium for cassava was prepared following protocol in 2.9.4. For acclimatization, plantlets were sub-cultured in CBM agar with lower gelrite percentage (2-2.5 g/L (w/v)) to minimize roots damage when transferring plant material from agar to soil.

8 to 10-day-old cassava *in vitro* culture was gently removed from agar medium by knocking the bottom of container on hard material (bench) to disrupt the agar and picking up the plantlet using forceps. Please note that using plant material older than 10 days could potentially reduce the survival rate of the plant as it has developed longer roots and more leaves that need to be trimmed which is harmful to plants in the acclimatization process. After removing from agar medium, the roots were washed with luke-warm tap water for 1-2 min. The plantlets were then laid on paper towel in a tray and were sprayed frequently with water to keep moist while transferring other plantlets. The plantlets were planted into the soil with great care by making a hole first in the soil and compressing it afterwards. The tray was covered with a transparent lid and was sealed to maintain high humidity inside the tray for 6 days. The lid was then slightly opened, leaving 1-2 cm air gap to let the plants adapt with the air outside. A week after, the lid was opened fully and the plants grown in the growth room for another 1-2 weeks before transferring them to the glass house.

2.13.8.3. Transfer of cassava plant to soil in the glasshouse

Well-grown plantlets were transferred to the glasshouse under controlled conditions of 28-30ºC, >50% relative humidity and 16 h daylight. About one week after the transfer, the plants were moved into 1-l pot (B.E.F growers pot no. 5 with 13 cm-upper diameters, 8 cm-lower diameters and 12 cm-high) containing the same medium composition. The plants were watered every day or 2 days until harvest. A total of 18 lines including 16 transgenic lines and 2 wild type lines were grown in the glasshouse and each line consisted of 35 plants for experimental replication.

2.13.9. Harvesting cassava

2.13.9.1. Measurement of agronomic traits

Besides PPD assessment, there were several agronomic traits measured in this study including plant height (cm), number of roots and weight of roots per plant (g). All measurements for each line were done on 3 biological replicates and each replicate consists of 3 plants.

6-month-old cassava plants were harvested and the roots were briefly washed in the glasshouse, the height was measured (cm), and the stem cut before transferring to the lab. The roots were immersed in tap water for 5 min to remove the remaining soil. The roots were then cut off the stem, counted and their weight (g) measured.

2.13.9.2. Preparation of cassava root for PPD experiment

Similar sized roots were selected for PPD evaluation in order to reduce variation results due to size differences. Selected roots were then immersed in 10% (v/v) bleach solution for 5 min to minimize bacterial and fungal contamination during the PPD evaluation.

2.13.9.3. Induction and measurement of cassava PPD

Cassava PPD was induced using the sliced method (Buschmann et al.,

2000). The roots were sliced about 10-15 mm thick and placed on a Petri dish containing dry filter paper (Whatman). The plates were then incubated in a chamber with the lid on under semi-controlled condition at 25±2 ºC, dark, no controlled relative humidity. PPD evaluation was carried out with 3 biological replicates and 3 technical replicates. Samples were observed at day 0, 2, 4, 6 and 8. The dehydrated part of sliced roots were removed (about 1-2 mm thick) and the roots were photographed (Pentax K20D) under a consistent camera set up (Exposure time: 1/180 sec, ISO-speed: 400, Aperture: f/5.6, Focal length: 50, Metering mode: Center Weighted Average, Flash mode: OFF, White balance: manual).

Cassava PPD level was determined according to root discoloration score measured by MatLab-based software (Vanderschuren et al., 2014). The software measures PPD level of selected areas of discoloured root (parenchyma tissue) by converting the colour images of the root to grey scale and analysing its frequency distribution of the root discoloration to generate a histogram with two different set of values figured as quantile 97.5% and 2.5% values. The PPD score was then calculated based on these values following the formula below:

$$PPD\ score\ = \frac{Quantile\ 97.5\% - Quantile\ 2.5\%}{Quantile\ 97.5\%}$$

PPD response is then expressed in % discoloration (PPD score x 100). To assess the potential delay of PPD in transgenic lines, we first calculated PPD rate at day 6 for each line (% discoloration day^{-1}) according to (Moyib et al., 2015) and then determined the time required by transgenic lines to reach the % discoloration of the wild type at the same day as an estimated delayed PPD time using the following formula.

$$Delayed\ time\ (DT) = \frac{PPDn\ (WT) - PPDn\ (transgenic\ lines)}{PPDn\ rate\ transgenic\ line\ (day^{-1})}$$

Where, DT: estimated delayed PPD time or time required for transgenic lines reaching PPD score (% discoloration) of the wild type at the same day, PPDn: % discoloration at selected PPD time point.

2.13.10. Analysis of gene expression level by RT-qPCR

Analysis of expression level of targeted gene(s) in the selected lines was performed on the Applied Biosystems 7900HT Fast Real-Time PCR System using Fast SYBR® Green I dye (Applied biosystems) for the detection of double-stranded DNA. The experiment was set up according to guidance from manufacturer (Applied biosystems).

2.13.10.1. Preparation of RNA and cDNA template

Ensuring good quality of RNA requires the A260/A280 to be greater than 1.9, and amounts of RNA are the key to succeeding qPCR analysis. Total RNA was isolated from cassava roots over a PPD time-course at day 0, 2, 4, 6 using the 2-day-CTAB protocol described in 2.10.3. Isolated total RNA was purified using a kit from Ambion, Life Technology and was quantified using the NanoVue.

cDNA synthesis was done using High Capacity cDNA Reverse Transcriptase Kit Reverse Transcriptase Kit (Applied Biosystem). 100-150 ng of purified RNA was added to the master mix (table 2.8) and the mixture was then incubated on a PCR machine with the following set-up: 25 ºC for 10 min, 37 ºC for 2 hours, 85 ºC for 5 min and 4 ºC forever (holding time). The cDNA was then checked for its quality using house-keeping gene primers, Ubq10 by PCR with the following reaction condition: 95ºC for 3 min, 95 ºC for 30 sec, 58 ºC for 30 sec, 72 ºC for 20 sec, 72 ºC for 5 min and 12 ºC forever (holding time) with 30 cycles. The PCR products were then run on 1.2% (w/v) gel agarose and visualised using an UV transilluminator.

Table 2.8. Composition of a 20 µL-master mix for cDNA synthesis.

No	Component	Volume (µL)
1	10x Buffer	2
2	100 mM dNTP mix	0.8
3	Random primer (10x RT)	2
4	Oligo primer (50 µM)	1
5	Reverse transcriptase (50 U/µL)	1
6	RNA sample (20 ng/ µL)	10
7	DW	3.2

2.12.10.2. Primers for qPCR and relative standard curve experiment

Several primers pairs had been designed for qPCR analysis (see 2.5.10). Relative standard curve experiment was performed to test the efficiency of each primer pair before using them for the analysis. In the relative standard curve experiment, cDNA sample was prepared in five different concentrations (1x, 0.2x, 0.04x, 0.008x and 0.0016x), in order to create a standard curve. Before running the experiment, the qPCR reaction was set up using StepOne Software V2.3 (Applied Biosystems, UK).

1x PCR master mix containing 10 µL of fast SYBR® green master mix, 0.4 µL of forward primer (200 nM), 0.4 µL of reverse primer (200 nM), and 8.2 µL of DW was prepared in a reaction plate (Applied Biosystems, UK). 1 µL of cDNA template was added into the mixture and the plate was sealed using optical adhesive covers (Applied Biosystems, UK). All preparation was done on ice until it was ready to be run on the machine. The plate was centrifuged at 2,000 rpm, RT for 2 min to remove any bubbles. The bubbles could also be removed by flicking the bottom of the plate and then centrifuging again. Once the plate was ready, it was placed on the sample holder of the machine. The analysis was run using the program for the

relative standard curve experiment that had been previously created with the following reaction conditions: 95 °C for 30 sec, 94 °C for 20 sec, 60 °C for 20 sec and 72 °C for 20 sec with 40 cycles in total, 95 °C for 15 sec and 40 °C for 2 min. The results were then analysed using StepOne Software V2.3. Primers pairs with the highest efficiency were used for comparative C_T experiment.

2.13.10.3. Comparative C_T experiment

The PCR master-mix was prepared in a reaction plate using the same preparation as for the standard curve experiment. For the comparative C_T experiment, cDNA template was used directly without any dilution. The analysis was run following program for comparative C_T experiment that had been previously created with the following reaction condition: 95 °C for 30 sec, 94 °C for 20 sec, 60 °C for 20 sec and 72 °C for 20 sec with 40 cycles in total, 95 °C for 15 sec and 40 °C for 2 min. The results were then analysed using StepOne Software V2.3.

2.13.11. Biochemical analysis of selected transgenic cassava

Four coumarins including scopoletin, scopolin, esculetin and esculin were extracted using ethanol and were analysed by LCMS.

Preparing standard solution for standard curve

Standard solutions of four coumarins were prepared in 10% (v/v) Methanol at various concentrations (mL^{-1}); 2,000 μg, 1,000 μg, 500 μg, 250 μg, 125 μg, 62.5 μg and 31.25 μg. Scopoletin has low solubility in lower percentages of either methanol or ethanol, hence an organic solvent, DMSO, was added to the solution in order to dissolve scopoletin completely.

***Preparing sample*:** 500 mg of cassava root was ground in a non-sterile mortar with liquid N_2 to a fine powder. The powder was transferred into a 5 mL screw-cap glass vial (Fisher). 2.0 mL of 100% (v/v) Ethanol (HPLC

grade) containing 100 ng/mL of the first internal control, 4-Methyl Umbelliferone (4-MU), and was incubated on a shaker (IKA-VIBRAX-VXR) at room temperature, 10 rpm, overnight or for 16 hours. The extract/liquid phase was filtered through a 0.45 µm PES syringe filter (Thermo Fisher) and 1.2 mL of filtrate was transferred into a 2 mL mL screw-cap microfuge tube (Molecular BioProducts). To evaporate the extraction solvent, the filtrate was then vacuum-concentrated in a Savant SpeedVac vacuum concentrator (Thermo Fisher) for about 3-4 h or until the solvent had completely evaporated. A Dried extract was then reconstituted with 200 µL of 10% (v/v) MeOH-HPLC grade containing 1 µg m/L scoparone as the second internal control and was stored at 4℃ for few hours or overnight to re-dissolve the extract. The re-dissolved extract was vortexed vigorously and centrifuged at 11,337 g for 5 min to spin down any unwanted particles such as starch. 150 µL of the extract was transferred to a 300 µL screw-cap vial (Waters) and sent for biochemical analysis using LC-MS to the Department of Pharmacy and Pharmacology, University of Bath.

Chapter 3. Identification and characterisation of scopoletin glucosyltransferase and scopolin beta-glucosidase gene-families in cassava

Chapter 3. Identification and characterisation of scopoletin-glucosyltransferase and scopolin-beta-glucosidase gene-families in cassava

3.1. Introduction

Scopoletin, which is synthesised *de novo* via phenylpropanoid metabolism and released from its glycone, scopolin, plays a key role in cassava postharvest deterioration, known as postharvest physiological deterioration (PPD). Oxidation of scopoletin by peroxidase compounds such as hydrogen peroxide (H_2O_2) forms a blue complex causing discoloration of the root (Wheatley and Schwabe, 1985). Recently, the inhibition of the genes for a key enzyme; feruloyl CoA 6′-hydroxylase in the biosynthesis of scopoletin significantly reduced PPD (Liu et al., 2017). Homeostasis of scopoletin in the cells is regulated by interconversion of scopoletin and scopolin, which indicates that this step may also play important role in the PPD response. The interconversion of scopoletin and scopolin involves two reactions: first is glucosylation of scopoletin catalysed by scopoletin- glucosyltransferase (scopoletin-GT) that converts scopoletin to its glycone, scopolin; and de-glucosylation of scopolin catalysed by scopolin beta-glucosidase (scopolin-BG) that converts scopolin to scopoletin. However, the role of this interconversion in cassava PPD is unclear and the genes for this step are not well studied in cassava. In this study, we aimed to disrupt this interconversion step by modifying the expression of the corresponding genes through RNA interference (RNAi) and overexpression to understand better their role on cassava PPD. These approaches could alter the accumulation of both compounds, thereby affecting the PPD response in cassava.

Since the specific cassava genes for both enzymes are not studied yet, the first strategy was to search for homologous genes encoding scopoletin-GT

and scopolin-BG in the cassava genome using reference genes, which was the main aim of this chapter. As scopoletin and scopolin accumulation have been identified in cassava roots (Bayoumi et al., 2010, Buschmann et al., 2000), we assume that the two enzymes and their genes exist in the cassava genome. However, the questions here were how can they be identified and what is their diversity in the genome? On the other hand, the genes for those enzymes have been studied in other plants such as *togt*1 gene for scopoletin-GT from tobacco (*Nicotiana tabacum*) and BGLU23 gene for scopolin-BG from *Arabidopsis thaliana*. Therefore, that work could facilitate the identification of homologous cassava genes and investigation of their biological functions in cassava. In this chapter, we also characterised identified homologous cassava genes and selected best candidate genes for functional studies.

3.1.1. Scopoletin-glucosyltransferase (scopoletin-GT) in plants

In plants, glycosylation is the last step in general phenylpropanoid metabolism that is responsible for the synthesis of various secondary metabolites such as flavonoids, lignin and coumarins including scopoletin, many of which are accumulated as glycosides (Lanot et al., 2006). Glycosylation is regulated by UDP-glycosyltransferase (UGT) and has the ability to change phenylpropanoid solubility, stability, toxic potential, as well as influence compartmentalisation and biological activity (Le Roy et al., 2016).

Glucosyltransferases (GTs) are a highly divergent, polyphyletic, and multigene family (Mackenzie et al., 1997). Some plant GT families have been classified and are available in the Carbohydrate-Active enZYmes (CAZY) database (Davies et al., 2005) and in a specific plant carbohydrate-active enzyme (PlantCAZyme) database (Ekstrom et al., 2014). The latter is a database built upon dbCAN (database for automated carbohydrate active enzyme annotation), containing data of 43790 CAZymes of 159

protein families from 35 plants species (including angiosperms, gymnosperms, lycophyte and bryophyte mosses) and chlorophyte algae with fully sequenced genomes (Ekstrom et al., 2014). In cassava, 481 GT genes that are classified into 41 gene families have been identified and are available on PlantCAZyme. However, to the best of our knowledge, there are no reported studies on specific cassava scopoletin-GTs in terms of number of genes and their gene family classification in the genome. This study would examine those aspects.

Glucosyltransferases (GTs) add activated sugars, mostly UDP-glucose, to low molecular weight substrates, such as major classes of secondary metabolites including phenolics, terpenoids, cyanohydrins (cyanogenic glycoside precursors), thiohydroximates (glucosinolate precursors) and alkaloids. Glycosylation enables the storage of potent toxic and deterrent chemicals in inactive forms at high concentration, while simultaneously allowing the plant to rapidly release chemicals or defense compounds towards microbial attack or other environmental stresses. The glycosylation reaction takes place in the cytosol before the products (glycosides) are transported to and stored in the vacuole (Vogt and Jones, 2000).

Studies on several members of the glycoside family including anthocyanins, the glucosinolates and cyanogenic glycosides have shown the importance of glycosylation in the stabilisation of secondary metabolites. For instance, in case of cyanogenic glycosides, the attachment of a glucose moiety is required to stabilise and prevent spontaneous degradation to cyanide, aldehydes or isothiocyanates (Vogt and Jones, 2000, Kahn et al., 1997). Similarly, in the biosynthesis of scopoletin, glucosylation, which is regulated by scopoletin glucosyltransferases (scopoletin-GTs), is believed to protect scopoletin against cellular oxidases as well as improve its solubility in the cell (Chong et al., 1999). Scopoletin (6-methoxy-7-hydroxycoumarin) is a highly reactive phenolic hydroxyl and is a known reactant of peroxidases *in*

vitro (Marquez and Dunford, 1995, Chong et al., 2002).

The biological role of scopoletin-GTs in plants has been investigated in tobacco. The tobacco glucosyltransferase (TOGT) genes encoding scopoletin glucosyltransferase (UDP-glucose:scopoletin *O*-β-D-glucosyltransferase, EC. 2.4.1.128) is up-regulated by salicylic acid (SA) and during the hypersensitive response (HR), additionally it accepts hydroxycinnamates and hydroxycoumarins, particularly the 6-methoxy-7-hydroxycoumarin scopoletin, as substrates *in vitro* (Horvath and Chua, 1996, Fraissinet-Tachet et al., 1998). Down-regulation of TOGT (*togt*1) using antisense constructs led to decreased not only of scopolin, but also unexpectedly of scopoletin in the *togt*1-down-regulated transgenic lines. Scopoletin was expected to accumulate through its biosynthetic pathway when its conversion to scopolin was inhibited (Figure 3.1). However, tobacco mosaic virus (TMV)-uninfected transgenic lines showed twofold decrease in scopoletin and scopolin levels compared with control plants, whilst in TMV-infected transgenic tobacco, scopoletin and scopolin levels were threefold to fourfold lower than that obtained in controls (Chong et al., 2002).

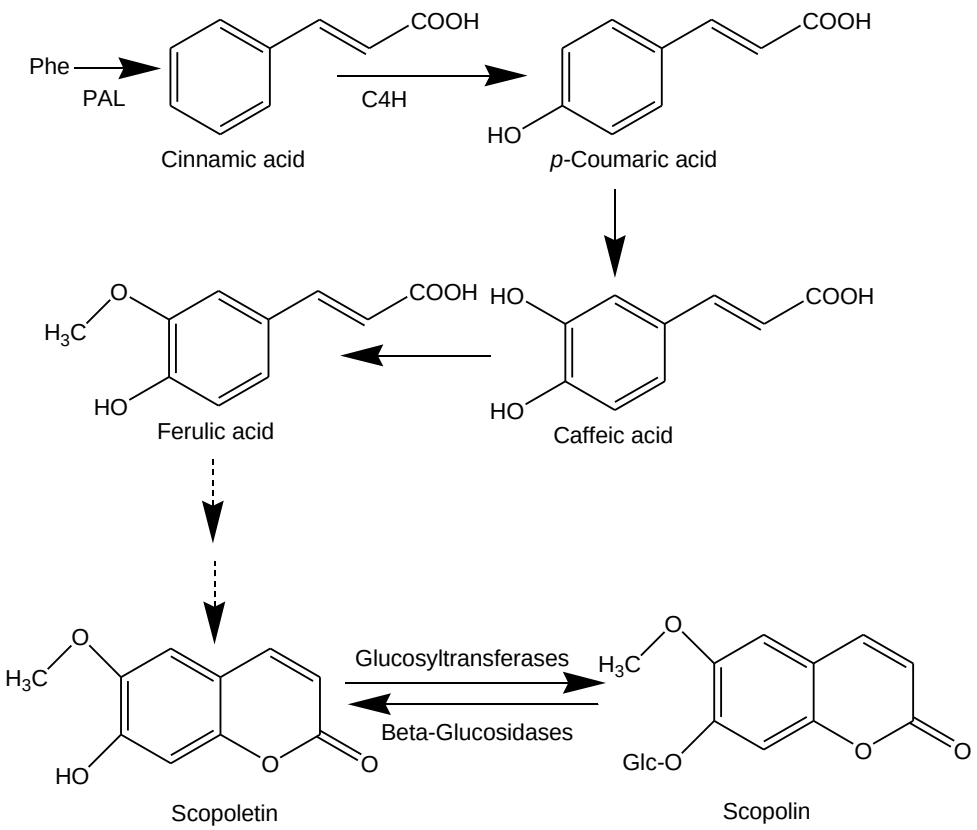


Figure 3.1. Biosynthesis pathway of scopoletin and scopolin in plants. The figure was re-drawn and slightly modified from (Chong et al., 2002).

There are three hypotheses suggested by Chong et al (2002) to explain this unexpected reduced scopoletin content. Firstly, it is known that phenolic compounds cannot accumulate to high levels as aglycones in plant cells because they are very reactive and subject to oxidation. Therefore, compromising scopoletin glucosylation could enhance its degradation by cellular oxidases. Secondly, one part of the scopoletin pool may arise from the hydrolysis of its glucoside form, scopolin by beta-glucosidase. Thirdly, inhibition of TOGT could alter metabolic flux towards scopolin, resulting in the feedback inhibition of upstream enzymes of the biosynthesis of scopoletin. The reduction of scopoletin and scopolin in the TOGT-transgenic lines also resulted in decreasing blue fluorescence in the cells around TMV infections, suggesting that scopoletin and scopolin are major compounds responsible for the blue fluorescence observed under the UV

light (Chong et al., 2002, Costet et al., 2002). This result suggested the possibility that similar manipulation of the cassava gene(s) homologous to *togt*1 gene could also alter scopoletin and scopolin contents in transgenic cassava roots, thereby affecting the PPD response.

3.1.2. Scopolin beta-glucosidase (Scopolin-BG) in plants

Beta-glucosidases (E.C.3.2.1.21) belong to the glycosyl hydrolase (GH) family that is widely distributed in all domains of living organisms. These enzymes regulate de-glycosylation of glycosides in phenylpropanoid metabolism and play an important role in hydrolysing the glycosidic bond between sugars and a non-sugar moiety (Ahn et al., 2010, Cairns and Esen, 2010). Beta-glucosidases have diverse biological functions not only in plants but also in Archaea, Eubacteria, and Eukaryotes.

In plants, beta-glucosidases play roles in lignification, catabolism of cell wall oligosaccharides, defense, phytohormone conjugate activation, and scent release, as well as in both sides of plant–microbe and plant–insect interactions. The roles of these enzymes are supposed to be controlled by their substrate-specificities, their tissue and subcellular localisation, and the conditions under which they are exposed to their physiological substrates (Cairns and Esen, 2010). Most of beta-glucosidases are substrate-specific although they tend to have similar molecular weight (MW) between 55 to 65 kDa, optimum pH between 5 to 6 and are evolutionary related (Esen, 1993, Henrissat, 1991).

Information on the GH family is available in the Carbohydrate-Active enZYme (CAZY) database (Davies et al., 2005) and a specific PlantCAZyme database (Ekstrom et al., 2014). Based on their amino acid sequence similarities, beta-glucosidases have been classified into several glycosyl hydrolase (GH) families; GH1, GH3, GH5, GH9 and GH30 and GH116. Most of beta-glucosidases belong to the CAZY GH1 family that

have the ability to catalyse reverse hydrolysis and transglycosylation reactions, resulting in the synthesis of oligosaccharides and alkyl polyglucosides (Cairns and Esen, 2010, Rouyi et al., 2014).

In cassava, the most studied beta-glucosidase is cyanogenic-beta-glucosidase (linamarase). It possess $(\beta/\alpha)_8$ barrel structure and is classified into the family-1 of glycosyl hydrolase (GH1) that can transfer a non-reducing glucosyl group from a glycoside or carbohydrate to water (hydrolysis) or alcohol (transglycosylation) via a retaining mechanism (Cairns and Esen, 2010). The cyanogenic beta-glucosidase (linamarase) had shown to have high synthetic ability but low hydrolytic activity (Svasti et al., 2003, Guo et al., 2015). Many beta-glucosidases show very specific activity towards the aglycone moiety of their natural substrate; therefore, identifying the natural substrate(s) of individual beta-glucosidases would be beneficial in analysing their biological functions, including in cassava.

Scopolin beta-glucosidase is a type of scopolin-substrate specific enzyme that has ability to transfer a glucose molecule from scopolin to release scopoletin as a plant defense compound. In cassava, the specific genes for this enzyme have not been studied yet; therefore, we used identified gene from other plants to search for homologous gene(s) in cassava. In Arabidopsis, three scopolin-hydrolysing beta-glucosidases (At1g66270-BGLU21, At1g66280-BGLU22, and At3g09260-BGLU23) were isolated from roots and their glucoside-hydrolysing activity was studied in insect cells (Ahn et al., 2010). The three beta-glucosidases showed the same pattern of specific hydrolytic activity for the natural substrate scopolin, together with some activity towards substrates that are structurally similar to scopolin, such as esculin and 4-MU-glucoside. However, recombinant BGLU23 showed the highest scopolin-hydrolysing activity among three BGLUs. Therefore, the BGLU23 was used as a reference gene to identify homologous genes in the cassava genome.

3.2. Aims of study and research strategies

This chapter aimed to identify genes encoding scopoletin-glucosyltransferase (scopoletin-GT) and scopolin-beta-glucosidase (scopolin-BG) in the cassava genome and select the best candidates for functional studies using Arabidopsis T-DNA insertion lines and cassava.

The following strategies were used to achieve the aims of the study in this chapter:

1. Identify homologous genes encoding scopoletin-glucosyltransferase and scopolin-beta-glucosidase in the cassava genome through BLAST searches using the reference genes; *togt*1 and BGLU23.
2. Analyse the evolutionary relationship of identified cassava scopoletin-glucosyltransferase and cassava scopolin-beta-glucosidase using phylogenetic trees in order to assist the identification of the cassava genes that are most closely related to the reference genes.
3. Characterise selected cassava genes using RT-PCR with gene specific primers to confirm their tissue specificity prior to complementation studies in Arabidopsis T-DNA insertion lines and cassava.

3.3. Results: Identification, distribution and gene expression profiles of scopoletin-glucosyltransferase (scopoletin-GT) and scopolin-beta-glucosidase (scopolin-BG) gene families in cassava.

Identification of homologous genes for scopoletin-GT and scopolin-BG was done not only in cassava but also in some other plants, such as *Ricinus communis, Arabidopsis thaliana* and *Oryza brachyantha.* Identifying homologous genes in Arabidopsis helped to identify Arabidopsis T-DNA insertion lines that could be used in complementation studies.

3.3.1. Cassava scopoletin-GTs homologous to *togt*1 belong to Glucosyltransferase family-1 (GT1)

The predicted amino acid sequence of the reference gene, *togt*1 (NCBI reference gene: AAK28303.1), was used to identify homologous genes in the cassava genome as this represents the functional part of the target enzyme. Using BLAST search on Phytozome database, we identified 164 cassava genes homologous to *togt*1 distributed across 28 different locations in the genome (Table 3.1).

Table 3.1. Identified cassava scopoletin-glucosyltransferase genes.

Program TBLASTN 2.2.26+			Program TBLASTN 2.2.26+		
No	Defline/location in the genome	Number of target hit (gene)	No	Defline/ location in the genome	Number of target hit (gene)
1	Chromosome10	14	16	Chromosome13	14
2	Chromosome05	15	17	Scaffold00727	01
3	Chromosome07	19	18	Scaffold00414	01
4	Chromosome09	05	19	Chromosome02	08
5	Scaffold00991	02	20	Chromosome04	06
6	Chromosome08	07	21	Scaffold01218	01
7	Chromosome01	18	22	Chromosome15	01

8	Chromosome12	16	23	Scaffold01003	01
9	Chromosome16	02	24	Scaffold01142	01
10	Chromosome17	01	25	Scaffold04975	01
11	Chromosome14	05	26	Scaffold02010	01
12	Chromosome06	07	27	Scaffold11237	01
13	Chromosome03	10	28	Scaffold02360	01
14	Chromosome11	03		Total	164
15	Scaffold00867	02			

BLAST detects local alignments that start with a small set of three amino acids or nucleotides in a specific order called the “query word”, between sequences that match the best. Then, each query is scored to determine which database is “in the neighbourhood” or the most comparable to the reference gene (Porterfield, 2014). The identified cassava genes showed different features, which are represented by amino acid sequence alignment similarity (score) and the significance of their alignments (E-value). The score describes the quality of an alignment, which means the higher score, the higher the similarity. While, E-value is an expected value of chance alignments, which means the smaller the E-value, the more significant the score is (Gonze, 2008).

In order to identify the best candidates for functional analysis, we did a preliminary screening of the 164 homologous cassava genes based on their score, E-value threshold (10E-10) (Wägele et al., 2011), and also functional annotation as described on the Phytozome database. Twenty-two out of 164 were selected with the score ranges from 675.7 to 80.6, E-value ranges from 0.0 to 4.3E-13 and with Glucosyl/Glucuronosyl transferases activity (Table 3.2). We found some identified genes still had no functional annotation in the description section on the Phytozome database, which might indicate that they were pseudogenes. Using the same BLAST search method, we also identified the *togt*1-homologous genes in different plants

species including *Ricinus communis, Arabidopsis thaliana,* and *Oryza brachyanta*, in order to see the diversity of the GT genes in other plants. The evolutionary relationship between reference gene, 22-selected cassava genes and selected homologous genes from other plants were analysed using phylogenetic tree to identify candidate cassava genes that were most closely related to the *togt*1. A new database of plant carbohydrate-active enzyme (PlantCAZyme) (Ekstrom et al., 2014) enabled us to identify the gene family of the cassava scopoletin-GT genes. The PlantCAZyme database revealed that the 22-selected cassava scopoletin-GT genes belong to the family-1 of glucosyltransferase (GT1) (Table 3.2).

Table 3.2. Selection of the 22 genes based on degree of amino acid sequences similarity.

No	Defline	Score	E-Value	Gene family (PlantCAZyme)	Description	Alias	Sequences
1	Chromosome10	675.7	0.0	GT1	PTHR11926//PTHR11926:SF347 - GLUCOSYL/GLUCURONOSYL TRANSFERASES	cassava4.1_029076m.g	Genomic: 1458 CDS: 1458 Protein: 485
2	Chromosome10	675.7	0.0	GT1	PTHR11926//PTHR11926:SF190 - GLUCOSYL/GLUCURONOSYL TRANSFERASES	cassava4.1_023510m.g	Genomic: 1775 CDS: 1464 Protein: 487
3	Chromosome10	675.7	0.0	GT1	PTHR11926//PTHR11926:SF246 -GLUCOSYL/GLUCURONOSYL TRANSFERASES	cassava4.1_034435m.g	Genomic: 1583 CDS: 1464 Protein: 487
4	Chromosome10	675.7	0.0	GT1	PTHR11926//PTHR11926:SF190 - GLUCOSYL/GLUCURONOSYL TRANSFERASES	cassava4.1_006629m.g	Genomic: 1446 CDS: 1446 Protein: 481
5	Chromosome10	675.7	0.0	GT1	PTHR11926//PTHR11926:SF190 - GLUCOSYL/GLUCURONOSYL TRANSFERASES	cassava4.1_029292m.g	Genomic: 1446 CDS: 1401 Protein: 466
6	Chromosome10	653.3	0.0	GT1	PTHR11926:SF167 - UDP-GLYCOSYLTRANSFERASE 84A1-RELATED	cassava4.1_006627m.g	Genomic: 1446 CDS: 1446 Protein: 481
7	Chromosome07	455.7	5.3E-126	GT1	PTHR11926//PTHR11926:SF190 - GLUCOSYL/GLUCURONOSYL TRANSFERASES	cassava4.1_030942m.g	Genomic: 1446 CDS: 1446 Protein: 481
8	Chromosome07	455.7	5.3E-126	GT1	PTHR11926//PTHR11926:SF190 - GLUCOSYL/GLUCURONOSYL TRANSFERASES	cassava4.1_025417m.g	Genomic: 1467 CDS: 1467 Protein: 488
9	Chromosome07	455.7	5.3E-126	GT1	PTHR11926//PTHR11926:SF246 - GLUCOSYL/GLUCURONOSYL TRANSFERASES	cassava4.1_025170m.g	Genomic: 1647 CDS: 1437 Protein: 478
10	Chromosome05	452.1	6.4E-125	GT1	PTHR11926//PTHR11926:SF190 - GLUCOSYL/GLUCURONOSYL	cassava4.1_006741m.g	Genomic: 1675 CDS: 1434

					TRANSFERASES		Protein: 477
11	Chromosome05	452.1	6.4E-125	GT1	PTHR11926:SF271 - UDP-GLYCOSYLTRANSFERASE 74D1	cassava4.1_022886m.g	Genomic: 2962 CDS: 1398 Protein: 465
12	Chromosome05	452.1	6.4E-125	GT1	(M=6) PTHR11926//PTHR11926:SF299 - GLUCOSYL/GLUCURONOSYL TRANSFERASES	cassava4.1_007706m.g	Genomic: 1637 CDS: 1371 Protein: 456
13	Chromosome05	452.1	6.4E-125	GT1	(M=8) PTHR11926:SF271 - UDP-GLYCOSYLTRANSFERASE 74D1	cassava4.1_028982m.g	Genomic: 2868 CDS: 1281 Protein: 427
14	Scaffold00991	134.7	2.2E-29	GT1	(M=11) PTHR11926:SF248 - UDP-GLYCOSYLTRANSFERASE 73C7	cassava4.1_034384m.g	Genomic: 1470 CDS: 1470 Protein: 489
15	Chromosome01	131.1	2.7E-28	GT1	(M=11) PTHR11926:SF248 - UDP-GLYCOSYLTRANSFERASE 73C7	cassava4.1_006160m.g	Genomic: 1886 CDS: 1497 Protein: 498
16	Chromosome01	131.1	2.7E-28	GT1	(M=5) PTHR11926:SF102 - GLUCOSYLTRANSFERASE-LIKE PROTEIN-RELATED	cassava4.1_027442m.g	Genomic: 1565 CDS: 1356 Protein: 451
17	Chromosome09	102.3	1.3E-19	GT1	(M=11) PTHR11926:SF248 - UDP-GLYCOSYLTRANSFERASE 73C7	cassava4.1_033869m.g	Genomic: 1959 CDS: 1617 Protein: 538
18	Chromosome08	102.3	1.3E-19	GT1	(M=11) PTHR11926:SF248 - UDP-GLYCOSYLTRANSFERASE 73C7	cassava4.1_006691m.g	Genomic: 2462 CDS: 1473 Protein: 490
19	Chromosome08	102.3	1.3E-19	GT1	(M=4) PTHR11926:SF292 - UDP-	cassava4.1_007209m.g	Genomic: 2454 CDS: 1386

					GLYCOSYLTRANSFERASE 78D1-RELATED		Protein: 461
20	Chromosome12	96.9	5.6E-18	GT1	(M=2) PTHR11926:SF176 - UDP-GLYCOSYLTRANSFERASE 87A2	cassava4.1_007402m.g	Genomic: 1732 CDS: 1365 Protein: 454
21	Chromosome12	96.9	5.6E-18	GT1	(M=3) PTHR11926//PTHR11926:SF356 - GLUCOSYL/GLUCURONOSYL TRANSFERASES	cassava4.1_028194m.g	Genomic: 1473 CDS: 1410 Protein: 469
22	Chromosome14	80.6	4.3E-13	GT1	(M=3) PTHR11926:SF253 - UDP-GLYCOSYLTRANSFERASE 89B1	cassava4.1_034323m.g	Genomic: 1428 CDS: 1428 Protein: 475

Phylogenetic analysis revealed six homologous cassava genes were most closely related to *togt*1

Phylogenetic tree analysis of the 72 selected genes homologous to *togt*1 including 22 genes from cassava, 20 genes from *Arabidopsis thaliana*, 15 genes from *Ricinus communis*, and 12 from other plants including *Oryza brachyantha* showed that the cassava genes were divided into four big groups (Figure 3.2). This helped us in the selection of the homologous cassava genes that are most closely related to the *togt*1 gene. The tree revealed that six homologous cassava genes were found to be most closely related to *togt*1 as they clustered together with the *togt*1 in group 1. Interestingly, the majority of the members including cassava, *Arabidopsis thaliana* and *Ricinus communis* that cluster together were located on the same chromosome in their genome. An amino acid sequence alignments revealed that the six selected homologous cassava genes share between 53% to 63% amino acid sequence identity with *togt*1 (Table 3.3).

Candidate cassava scopoletin-GT for functional studies

The six most closely related cassava genes were then selected for the functional studies. Before using them in the functional studies, their expression was first analysed by RT-PCR using cDNA from leaf and root at different time points of PPD. The results showed only two, *MeSGT*1 and *MeSGT*6, of six tested genes were expressed in the root. *MeSGT*1 showed stronger and more consistent expression in the root over the PPD time course (Figure 3.3) while two cassava genes, *MeSGT*1 and *MeSGT*2 were expressed in leaf (Figure 3.4).

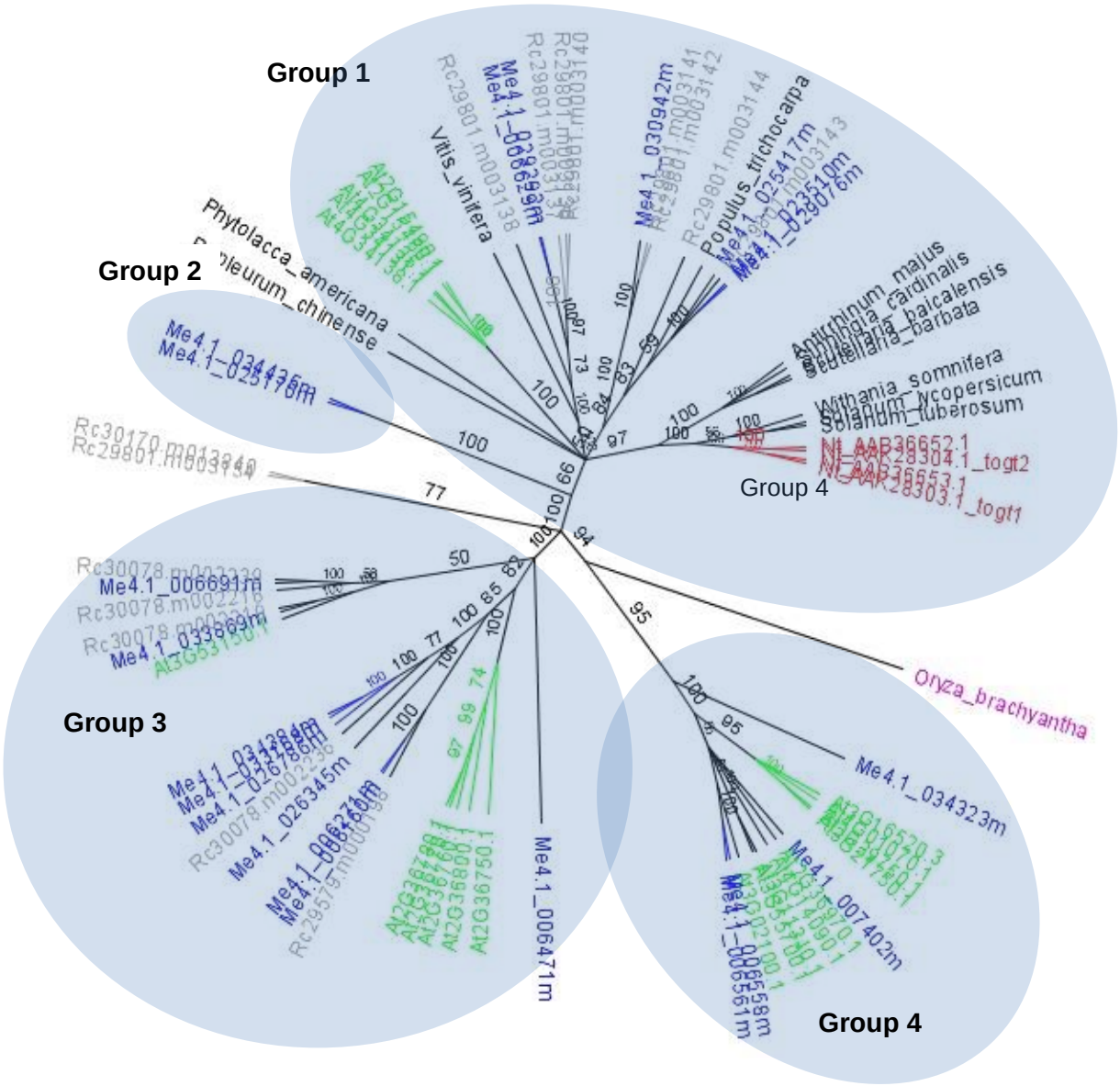


Figure 3.2. Unrooted phylogenetic tree of predicted 72 amino acid sequences of scopoletin-GT from various plants.

Samples including Tobacco, *Nicotiana tabacum*, cassava, *Manihot esculenta (Me)*, *Arabidopsis thaliana*, *Ricinus communis*, *and* other plants including *Oryza brachyanta*. The phylogenetic tree was built using Geneious tree builder; genetic distance model: Jukes-Cantor, tree building method: Neighbor-Joining, Resampling method: Bootstrap and number of replication: 1000.

Table 3.3. Identity of six selected homologous cassava scopoletin-GT genes closely related to *togt*1.

No	Cassava gene ID	Amino acid (residues)	% identity	Alias
1	Cassava4.1_006629	481	55	MeScopoletin-GT1 (*MeSGT*1)
2	Cassava4.1_029292	466	53	MeScopoletin-GT2 (*MeSGT*2)
3	Cassava4.1_030942	481	57	MeScopoletin-GT3 (*MeSGT*3)
4	Cassava4.1_025417	488	56	MeScopoletin-GT4 (*MeSGT*4)
5	Cassava4.1_023510	487	63	MeScopoletin-GT5 (*MeSGT*5)
6	Cassava4.1_029076	485	63	MeScopoletin-GT6 (*MeSGT*6)

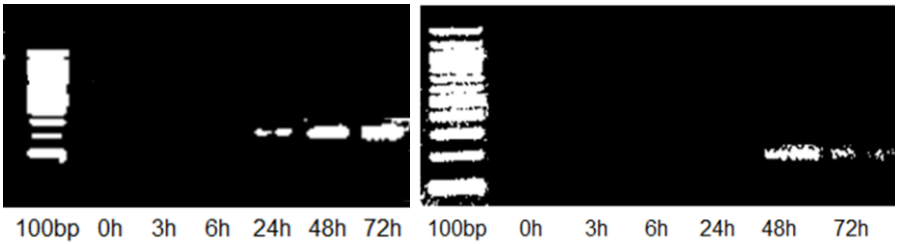


Figure 3.3. Expression profiles of *MeSGT*1 and *MeSGT*6 in cassava root over PPD time course confirmed by RT-PCR.

Figure 3.4. Expression profiles of six selected cassava genes homologous to the *togt*1 in cassava leaf. 1) *MeSGT*5, 2) *MeSGT*4, 3) *MeSGT*1, 4) *MeSGT*3, 5) *MeSGT*6, 6) *MeSGT*2.

Based on the expression profiles, the *MeSGT*1 was selected to create RNAi constructs for gene silencing studies in cassava. The two genes (*MeSGT*1 and *MeGT*6) share 61% identity in their amino acid sequences (Figure 3.4). Moreover, the RNAi constructs was designed to target both genes. In parallel, we did functional confirmation of the *MeSGT*1 gene in Arabidopsis to see whether this gene complements the Arabidopsis mutant.

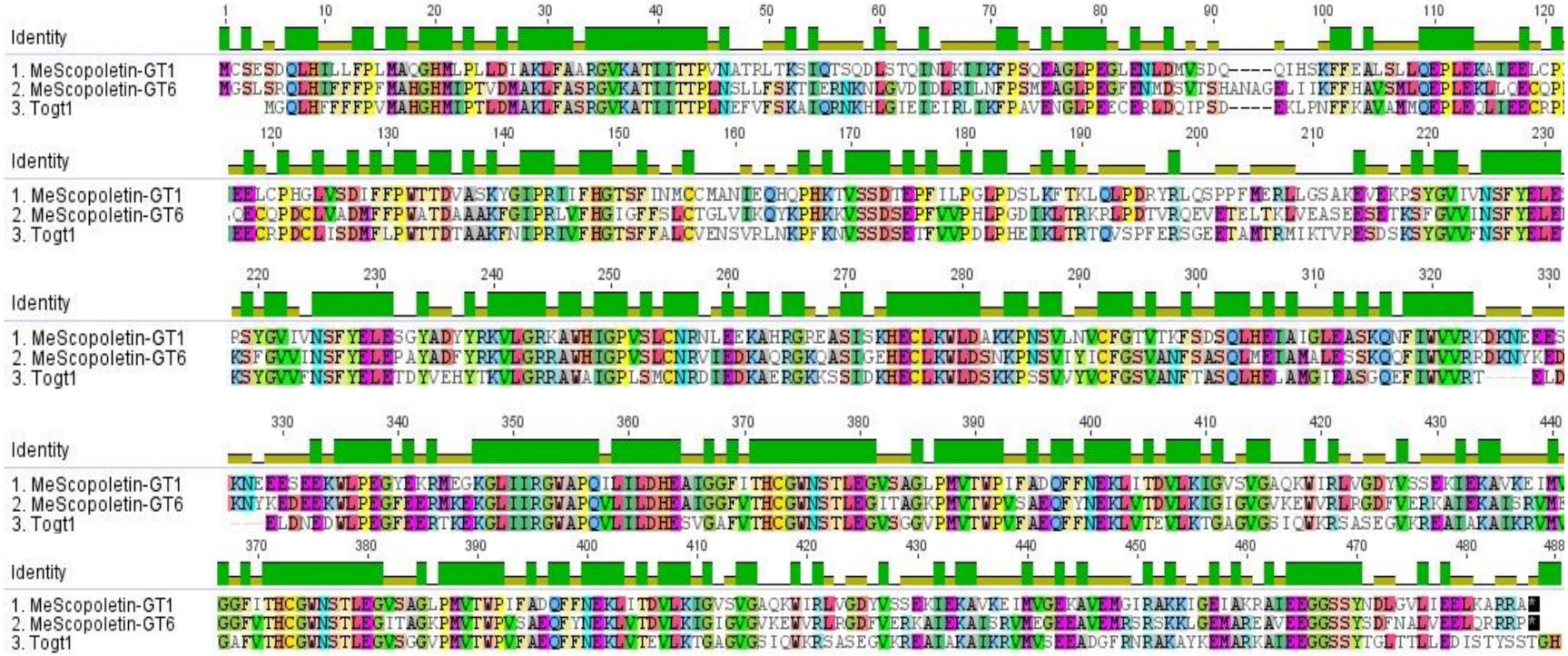


Figure 3.5. Multiple amino acid sequence alignment of two-root expressed GT gene; MeScopoletin-GT1, MeScopoletin-GT6 and *togt*1. Multiple alignment was done using Geneious Alignment program. Green: 100% identity, Greeny-brown: at least 30% and under 100% identity and Red: below 30% identity (Geneious).

3.3.2. Cassava scopolin-BG genes homologous to BGLU23 belong to glycosyl hydrolase family-1 (GH1)

BLAST search using the predicted amino-acid sequence of Arabidopsis scopolin-beta-glucosidase, BGLU23 (NCBI reference sequence: NP_187537.1) on Phytozome (Goodstein et al., 2012) identified 64 cassava genes homologous to the BGLU23, which are located on 16 different locations in the genome (Table 3.4).

Table 3.4. Identified cassava scopolin-BG genes homologous to BGLU23.

Program BLASTN 2.2.26+			Program TBLASTN 2.2.26+		
No	Defline/location in the genome	Number of target hit (gene)	No	Defline/location in the genome	Number of target hit (gene)
1	Scaffold00917	10	11	Scaffold01081	02
2	Scaffold02070	01	12	Chromosome16	01
3	Chromosome10	17	13	Chromosome12	01
4	Chromosome07	12	14	Chromosome17	02
5	Chromosome05	01	15	Chromosome13	01
6	Chromosome14	04	16	Chromosome09	01
7	Chromosome03	04		Total hits	64
8	Chromosome15	03			
9	Chromosome01	03			
10	Chromosome06	01			

Twenty-eight out of 64 homologous cassava genes encoding beta-glucosidase were selected based on their amino acid sequence alignment similarity (score), significance (E-value) and functional annotation on the Phytozome database with score ranges from 143.3 to 68.9, E-value from 5.9E-49 to 1E-10 and having glycosyl hydrolase activity as described on the Phytozome database. The PlantCAZyme database revealed that all the 28-selected homologous cassava scopolin-BG genes belong to glycosyl

hydrolase family-1 (GH1) (Table 3.5). Multiple alignments of their amino acid sequences revealed that all of them share low amino acid similarity between 34-46% identities with the BGLU23. Using the BLAST search method, we also identified genes homologous to BGLU23 in different plants species including *Ricinus communis, Arabidopsis thaliana,* and *Oryza brachyantha* to see the diversity of the genes homologous to the BGLU23 in other plants. All identified homologous genes from various plants were then analysed their evolutionary relationship using phylogenetic tree analysis.

Table 3.5. Selected 28 cassava genes encoding scopolin-BG based on their degree of amino acid alignment similarity.

No	Defline	Score	E-Value	Gene family (PlantCAZyme)	Description	Alias	Sequences
1	Scaffold00917	110.9	5.9E-49	GH1	Beta-glucosidase	cassava4.1_029035m.g	Genomic: 2705 CDS: 1479 Protein: 492
2	Scaffold00917	110.9	5.9E-49	GH1	Beta-glucosidase	cassava4.1_021468m.g	Genomic: 2844 CDS: 1593 Protein: 530
3	Scaffold02070	109.0	1.4E-46	GH1	Beta-glucosidase	cassava4.1_031399m.g	Genomic: 2749 CDS: 1527 Protein: 508
4	Chromosome10	109.0	3.2E-44	GH1	Glycosyl hydrolase	cassava4.1_032669m.g	Genomic: 2116 CDS: 1287 Protein: 428
5	Chromosome10	109.0	3.2E-44	GH1	Glycosyl hydrolase	cassava4.1_008514m.g	Genomic: 3755 CDS: 1560 Protein: 519
6	Chromosome10	109.0	3.2E-44	GH1	Beta-glucosidase	cassava4.1_029389m.g	Genomic: 4276 CDS: 1365 Protein: 454
7	Chromosome10	109.0	3.2E-44	GH1	Beta-glucosidase	cassava4.1_024320m.g	Genomic: 3746 CDS: 1557 Protein: 418
8	Chromosome07	123.6	1.2E-36	GH1	Beta-glucosidase	cassava4.1_027713m.g	Genomic: 3712 CDS: 1587 Protein: 528
9	Chromosome07	123.6	1.2E-36	GH1	Beta-glucosidase	cassava4.1_025199m.g	Genomic: 3498 CDS: 1539

							Protein: 512
10	Chromosome07	123.6	1.2E-36	GH1	Glycosyl hydrolase	cassava4.1_006309m.g	Genomic: 2922 CDS: 1614 Protein: 537
11	Chromosome07	123.6	1.2E-36	Not identified	Glycosyl hydrolase	Manes.07G027700	Genomic: 2918 CDS: 1629 Protein: 542
12	Chromosome07	123.6	1.2E-36	Not identified	Glycosyl hydrolase	Manes.07G027800	Genomic: 1548 CDS: 930 Protein: 309
13	Chromosome07	123.6	1.2E-36	GH1	Beta-glucosidase	cassava4.1_029195m.g	Genomic: 3470 CDS: 1659 Protein: 553
14	Chromosome07	123.6	1.2E-36	GH1	Beta-glucosidase	cassava4.1_021061m.g	Genomic: 3206 CDS: 1626 Protein: 541
15	Chromosome05	84.7	1.7E-34	GH1	Beta-glucosidase 1-related	cassava4.1_032290m.g	Genomic: 3145 CDS: 1506 Protein: 501
16	Chromosome05	84.7	1.7E-34	GH1	Beta-glucosidase 1-related	cassava4.1_006332m.g	Genomic: 3424 CDS: 1533 Protein: 510
17	Chromosome14	143.3	2.8E-34	GH1	Glycosyl hydrolase	cassava4.1_032229m.g	Genomic: 3023 CDS: 1533 Protein: 510
18	Chromosome14	143.3	2.8E-34	GH1	Glycosyl hydrolase	cassava4.1_005766m.g	Genomic: 3404 CDS: 1542 Protein: 513
19	Chromosome14	143.3	2.8E-34	GH1	Beta-glucosidase 43-related	cassava4.1_005890m.g	Genomic: 4673 CDS: 1530

							Protein: 509
20	Chromosome03	105.1	5.2E-32	GH1	Glycosyl hydrolase	cassava4.1_032622m.g	Genomic: 3545 CDS: 1617 Protein: 538
21	Chromosome03	105.1	5.2E-32	GH1	Glycosyl hydrolase	cassava4.1_026351m.g	Genomic: 3515 CDS: 1473 Protein: 490
22	Chromosome15	91.3	7.2E-30	GH1	Beta-glucosidase 41-related	cassava4.1_032518m.g	Genomic: 3353 CDS: 1515 Protein: 504
23	Chromosome15	91.3	7.2E-30	GH1	Beta-glucosidase 45-related	cassava4.1_031147m.g	Genomic: 3149 CDS: 1524 Protein: 507
24	Chromosome01	124.4	2.2E-28	GH1	Beta-glucosidase 40-related	cassava4.1_028433m.g	Genomic: 4102 CDS: 1521 Protein: 506
25	Chromosome01	124.4	2.2E-28	GH1	Beta-glucosidase 1-related	cassava4.1_005780m.g	Genomic: 3849 CDS: 1539 Protein: 512
26	Chromosome06	115.2	2E-25	GH1	Glycosyl hydrolase	cassava4.1_022953m.g	Genomic: 2775 CDS: 1563 Protein: 520
27	Chromosome16	81.3	1.4E-14	GH1	Beta-glucosidase 45-related	cassava4.1_005677m.g	Genomic: 9015 CDS: 1551 Protein: 516
28	Chromosome12	68.9	1E-10	GH1	Beta-glucosidase	cassava4.1_006336m.g	Genomic: 7157 CDS: 1476 Protein: 491

Phylogenetic analysis revealed that none of identified homologous cassava genes were closely related to BGLU23

Phylogenetic tree analysis of the 65 identified genes homologous to the BGLU23 including 28 genes from cassava (*Manihot esculenta* C.), 13 genes from *Ricinus communis,* 17 genes from *Arabidopsis thaliana,* and 7 genes from other plants showed that the identified homologous cassava genes were divided into five groups (Figure 3.6). Unfortunately, the phylogenetic tree showed that none of the 28-selected cassava genes cluster together with the reference gene; BGLU23. Therefore, we were not able to determine which of them was most closely related to BGLU23 hence it was not possible to select best candidate genes for gene silencing in cassava like scopoletin-GT gene.

The initial purpose of this search was to identify cassava scopolin-BG gene(s) homologous to BGLU23 with potential to knock-down expression of the corresponding gene(s) in transgenic cassava using an RNAi constructs. However, the sheer number of potential cassava candidate genes, their distance from Arabidopsis (BGLU23) and their clustering into diverse groups, suggested that identifying a single gene with the potential to target all members of this diverse gene family was unlikely. Therefore, an alternative strategy suggested itself, which was to over-express the scopolin-BG from Arabidopsis, BGLU23 in cassava with the purpose of accelerating the conversion of scopolin to scopoletin and thereby, altering the accumulation of both scopolin and scopoletin in the storage roots, subsequently potentially affecting PPD response.

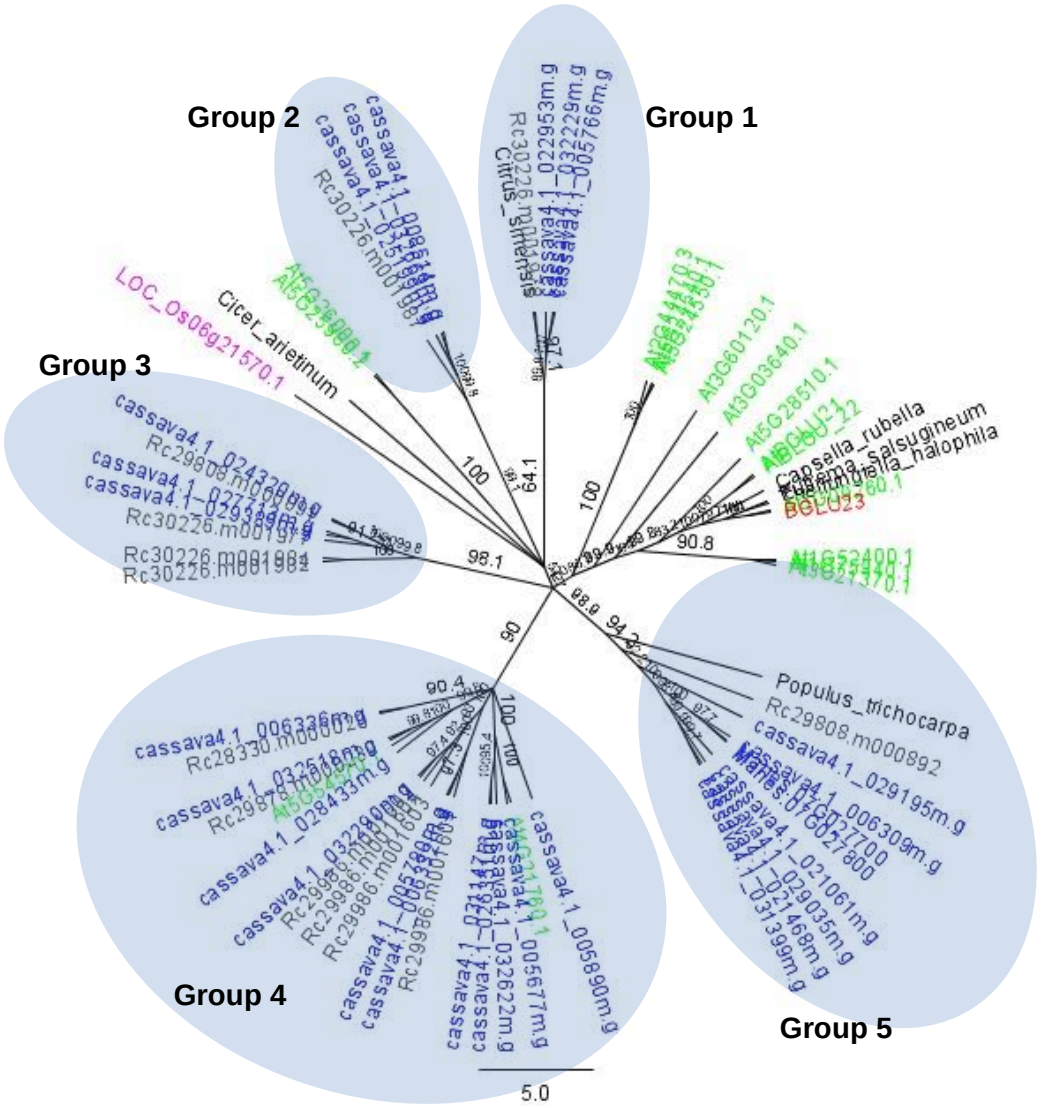


Figure 3.6. Unrooted phylogenetic tree of 65 protein sequences of scopolin-BG from various plants.

Samples consist of BGLU23 as reference gene, cassava, *Manihot esculenta (Me)*, *Arabidopsis thaliana*, *Ricinus communis*, and other plants. The phylogenetic tree was built using Geneious tree builder; genetic distance model: Jukes-Cantor, tree building method: Neighbor-Joining, Resampling method: Bootstrap and number of replications: 1000.

Candidate cassava scopolin-BG genes for functional studies using Arabidopsis T-DNA insertions lines

Because we could not identify the most closely related cassava gene to BGLU23, we chose a representative gene from each group randomly for the functional confirmation studies. Their expression was analysed using RT-PCR with cDNA from root. The results showed that only one tested cassava gene, *MeSBG*4, which is a member of group 3 in the phylogenetic tree, expressed in the roots at 48 h of PPD (Figure 3.7). More members of other groups had also been tested, but none of them were expressed in root cDNA. In addition, all gene specific primers had been tested first on genomic DNA to make sure that they are working. Rather than continue the search for further root-expressed genes it was decided to test the function of only *MeSBG*4 in Arabidopsis T-DNA insertion mutants.

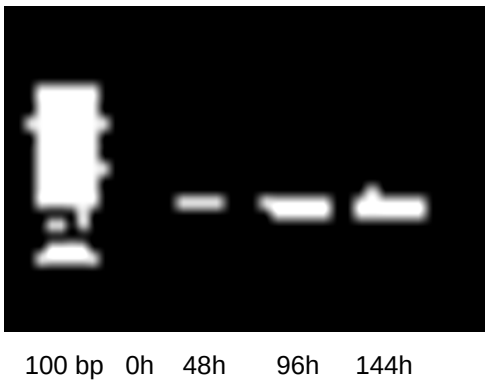


Figure 3.7. Expression of *MeSBG*4 gene encoding beta-glucosidase in cassava root sample at different time points of PPD, confirmed by RT-PCR.

3.4. Discussion

This chapter aimed to identify cassava genes encoding scopoletin-glucosyltransferase (scopoletin-GT) and scopolin-beta-glucosidase (scopolin-BG) using the reference genes, *togt*1 and GBLU23, respectively, and select best candidates for functional studies in Arabidopsis TDNA-insertion lines and cassava. Although information on both

glucosyltransferases (GTs) and beta-glucosidases (BGs) genes in cassava are currently available in the PlantCAZyme database (Ekstrom et al., 2014), no studies on specific genes for the scopoletin-GT and scopolin-BG enzymes in cassava reported. We found that cassava genes encoding scopoletin-glucosyltransferase (scopoletin-GT) homologous to *togt*1 and scopolin-beta-glucosidases (scopolin-BG) homologous to BGLU23 belong to large gene families.

Glucosyltransferases (GTs) are a highly divergent, polyphyletic, and multigene family (Mackenzie et al., 1997). Cassava scopoletin-GT is encoded by multiple genes that belong to super-family containing a hundred genes in the genome, which demonstrate the diversity of the cassava scopoletin-GT. Although it is not clear whether this encoding is due to a variety of enzyme structures or it is simply to rapid production of the enzyme under stress conditions (Dixon and Paiva, 1995), this encoding may be correlated with a broad enzyme activity, as was shown by two UDP-glucosyltransferases from cassava, UGT85K4 and UGT85K5, catalysing the last step in the biosynthesis of cyanogenic glucoside (Kannangara et al., 2011). This broad enzyme activity indicates that substrate specificities of some UDP-glucosyltransferases are defined by region-specific or region-selective features of the aglycones (Vogt and Jones, 2000). Current data on the PlantCAZyme (Ekstrom et al., 2014) show that in cassava, 481 GT genes that are classified into 41 gene families have been identified in which GT1 family is the largest gene family with a member of 153 genes or 31.8% of total GT genes identified in the cassava genome. We found that most of the selected homologous cassava genes belong to glucosyltransferase family-1 (GT1), six of which were the most closely related to *togt*1 as they cluster together in the phylogenetic tree and share amino acid sequences similarity between 53 and 63%. RT-PCR revealed *MeSGT*1 shows strong and more consistent expression in roots and leaves. Therefore, the *MeSGT*1 was chosen for the plasmid constructions including

overexpression and RNAi constructs for Arabidopsis and cassava transformation, respectively.

Furthermore, in terms of cassava genes encoding beta-glucosidase, this enzyme is also encoded by multiple genes in the genome. Beta-glucosidases belong to the glycoside hydrolase (GH) family that is widely distributed in all domains of living organisms (Cairns and Esen, 2010). Using data on PlantCAZyme, we found that most of the identified homologous cassava scopolin-BG genes belong to glycosyl hydrolase family-1 (GH1). These findings support Rouyi et al (2014) who found that most beta-glucosidase were included in the GH1 family. Cairns & Essen (2010) investigated that cassava cyanide beta-glucosidase gene also belongs to GH1 family. In cassava, 34 glycosyl hydrolase (GH) families containing 472 genes have been identified and available on PlantCAZyme database (Ekstrom et al., 2014). Unlike scopoletin-GT gene, none of the selected 28 homologous cassava scopolin-BG genes were closely related to the reference gene, BGLU23, which made it difficult to determine the best candidates for gene knock-down in cassava. Therefore, as an alternative strategy, we decided to overexpress full-length cDNA of the reference gene, BGLU23, in cassava in order to study the role of interconversion of scopoletin and scopolin in cassava PPD. This strategy could theoretically reduce scopolin accumulation and increase scopoletin levels in transgenic cassava root. However, earlier findings in tobacco showed that scopoletin levels were decreased when the conversion of scopoletin to scopolin was inhibited (Chong et al., 2002), which suggests that scopoletin cannot accumulate to high levels in the cells. Therefore, it might lead to another interesting result in cassava, in terms of scopoletin and scopolin accumulation that can affect PPD response.

For the functional confirmation of cassava genes in Arabidopsis T-DNA insertion mutant, we focused on one gene; *MeSBG*4 due to some difficulties

as described in the results section (see 3.3.2). Finding cassava genes that are most closely related to the reference gene, BGLU23, for gene knock down in cassava was the main issue at this stage due to their low amino acid sequence similarity. In a current BLAST search on Phytozome database, we noticed that the database has been updated with new identified cassava genes or re-annotation of the existing genes. We found some of identified homologous cassava genes are currently not available on the database while some have been updated their sequences. In addition, more information on genes families from several plant species including cassava and Arabidopsis is now available on the PlantCAZyme database (Ekstrom et al., 2014), which is useful for the re-identification of cassava scopolin-BG genes to obtain better candidates for functional study.

More fundamentally, our findings on the identity of both scopoletin-GT and scopolin-BG in cassava can be used as a starting point to explore more about the corresponding genes for future work. The functional confirmation of the selected cassava genes for both scopoletin-GT (*MeSGT*1) and scopolin-BG (*MeSBG*4) in Arabidopsis knock out lines will be discussed in chapter 4.

Chapter 4. Functional confirmation of cassava scopoletin-glucosyltransferase and scopolin-beta-glucosidase genes in *Arabidopsis thaliana*

Chapter 4. Functional confirmation of cassava scopoletin-glucosyltransferase and scopolin-beta-glucosidase genes in *Arabidopsis thaliana*

4.1. Introduction

The completion of Arabidopsis genome sequencing has made it possible to create loss-of-function mutations for all the genes as a tool for functional genomics analysis (Alonso et al., 2003). The availability of Arabidopsis knock out mutants for most genes in the genome has indeed broaden research in plant sciences to study functional genomics of other plants (Krysan et al., 2002). At least 225,000 independent knock out mutants have been generated by using transferred-DNA (T-DNA) insertional mutagenesis from *Agrobacterium tumefaciens* and been available in the electronic database at The Salk Institute Genome Analysis Laboratory (SIGnAL) (Alonso et al., 2003, Leicht and Cheng, 2009). The most identified lines are commercially available at The Nottingham Arabidopsis Stock Centre (NASC) or Arabidopsis Biological Resource Center (ABRC) at Ohio State University.

The identified homologous cassava genes for scopoletin-GT and scopolin-BG (Chapter 3) retrieved from the Phytozome database are described to have similar enzyme activity with the reference genes, *togt*1 and BGLU23, respectively. However, experimental evidence or published data on these specific genes in cassava are not yet available. Therefore, there is a need to confirm the function of the selected homologous cassava genes prior to the genetic modification in cassava, in order to avoid the possible off-target effects. In the previous chapter, we have selected cassava gene, *MeSGT*1, encoding scopoletin-GT and *MeSBG*4 encoding scopolin-BG for functional studies in Arabidopsis. The functional confirmation was carried out by overexpressing full-length cDNA of the selected cassava genes in

Arabidopsis T-DNA insertion lines using overexpression vector driven by either constitutive CaMV-35S or root-specific *StPATATIN* promoters. By expressing homologous cassava genes in Arabidopsis mutant, we would be able to see whether the selected cassava genes complement the mutant or not. Hence, the research question in this chapter was do the selected homologous cassava genes complement Arabidopsis T-DNA insertion lines? To be able to confirm this, we measured scopoletin and scopolin in samples of wild type, knock out lines and transgenic.

4.2. Aim of study and research strategies

The aim of this chapter was to confirm the function of the selected homologous cassava genes, *MeSGT*1 encoding scopoletin-GT and *MeSBG*4 scopolin-BG in Arabidopsis T-DNA insertion lines.

In order to achieve the aim of the study in this chapter, the following strategies were used:

1. Identify and characterise Arabidopsis T-DNA insertion lines in a gene of interest.
2. Create cassava scopoletin-GT and cassava scopolin-BG overexpression vectors using the GATEWAY cloning technology.
3. Transform Arabidopsis T-DNA insertion lines and select transgenic Arabidopsis lines.
4. Analyse the levels of scopoletin and scopolin in Arabidopsis wild type, T-DNA insertion lines and transgenic lines by LCMS.

4.3. Results

4.3.1. Identification and characterisation of Arabidopsis T-DNA insertion lines in a gene of interest

Two Arabidopsis T-DNA insertion lines, namely SALK_097487 for scopoletin-GT mutant and GABI-732F07 for scopolin-BG mutant were identified from Salk Institute Genomic Analysis Laboratory (SIGnAL) database for the study (Table 4.1). The T-DNA inserted into the exon in different chromosomes within the genome for each mutant.

Table 4.1. Identified T-DNA insertion lines for scopoletin-GT (Arabidopsis gene ID: At4g34131) and scopolin-BG (Arabidopsis gene ID: At3g09260).

No	T-DNA insertion line	Length (bp)	Location of insertion	Chromosome	Collection
1	SALK_097487	197	Exon	4	SALK Institute, USA
2	GABI-732F07	95	Exon	3	GABI-kat, Germany

The seeds of the Arabidopsis mutants were purchased from the Nottingham Arabidopsis Stock Centre (NASC). The seeds obtained from the company or collection centre are usually in a mixture or a population containing homozygous wild type, heterozygous mutant and homozygous mutant. Therefore, a genotyping analysis is required in order to identify homozygous mutant prior to the plant transformation.

PCR-based genotyping revealed homozygous Arabidopsis mutants

Screening of homozygous T-DNA mutant was done by PCR with specific T-DNA primers including left and right gene specific primers (LP and RP) and the left T-DNA border primer (LB), which were obtained from T-DNA primer

design at http://signal.salk.edu/tdnaprimers.2.html (Figure 4.1).

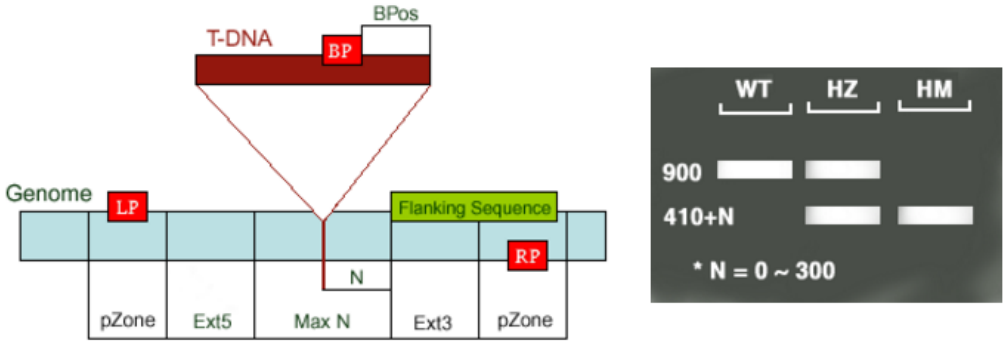


Figure 4.1. SALK T-DNA primer design. Three possible genotypes generated from PCR genotyping (SIGnaL). Note: N (Difference of the actual insertion site and the flanking sequence position, usually 0-300 bp), MaxN (Maximum difference of the actual insertion site and the sequence (300 bp)), pZone (Regions used to pick up primers (100 bp)), Ext5, Ext3 (Regions between the MaxN to pZone, reserved not for picking up primers), LP and RP (Left and Right genomic primer), BP (T-DNA border primer LB-the left T-DNA border primer), BPos (The distance from BP to the insertion site). Retrieved from http://signal.salk.edu/tdnaprimers.2.html.

PCR genotyping was carried out using genomic DNA isolated from 2-3-week-old rosette leaves and the reaction was run in two separate reactions set up: 1) with LP and RP primers and 2) with LB and RP primers. To determine the genotype of the mutants, two PCR products from LP+RP and LB+RP primers were combined (Figure 4.2).

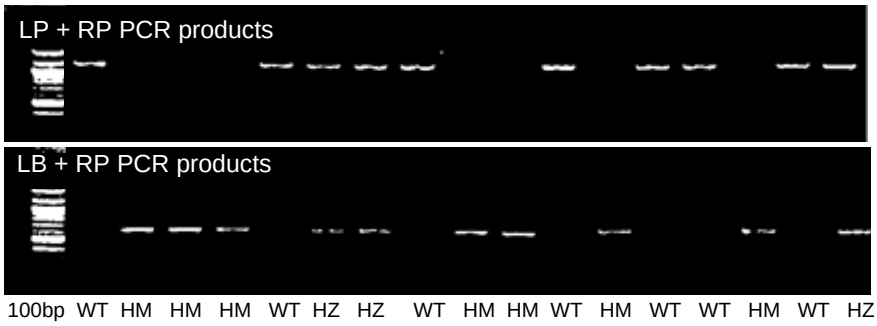


Figure 4.2. PCR genotyping of SALK_097487 line generate three genotypes. WT: homozygous wild type, HZ: heterozygous mutant and HM: homozygous mutant.

The percentages of wild type alleles and heterozygous mutants were relatively high, about 50-67%, although the seeds were labelled as

homozygous lines according to the description from the stock centre (NASC) where it was purchased from (Table 4.2).

Table 4.2. Variation of three possible genotypes in Arabidopsis T-DNA inserted mutants.

No	T-DNA insertion line	Homozygous mutant	Heterozygous mutant	Homozygous wild type
1	SALK_097487	49%	34%	17%
2	GABI-732F07	32%	26%	42%

Expression profile of mutated genes confirmed by RT-PCR

To confirm if the gene of interest had been knocked out, the expression of mutated genes in the homozygous mutants for both scopoletin-GT and scopolin-BG was analysed by RT-PCR using cDNA from roots. If the gene had been knocked out, there should be no expression detected on RT-PCR. DNA sample was included to make sure that there was no DNA contamination in the cDNA sample when running the analysis. The results showed that no expression was detected in both T-DNA insertion mutants (Figure 4.3).

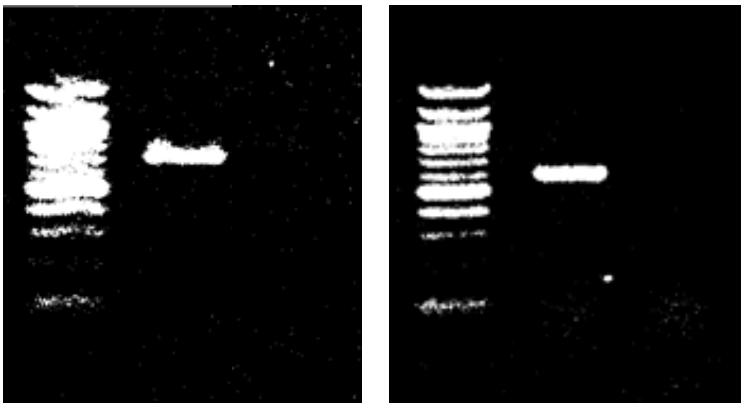


Figure 4.3. Gene expression of homozygous SALK_097487 and GK-732F07 was confirmed by RT-PCR.

Knocking out Arabidopsis scopoletin-GT gene reduced scopoletin and scopolin contents, while no significant reduction of both compounds in Arabidopsis scopolin-BG knock out line

In order to investigate further the effects of the insertion in each mutant, we measured scopoletin and scopolin contents in wild type as control, and T-DNA inserted mutants using LC-MS (Figure 4.4). The results showed that knocking out scopoletin-GT significantly reduced both scopoletin and scopolin contents in Arabidopsis SALK_097487 root samples. Scopoletin and scopolin were detected very low in leaves and no different among all samples including wild type and knock out lines. Moreover, scopoletin and scopolin accumulated considerably higher in wild type roots than leaves.

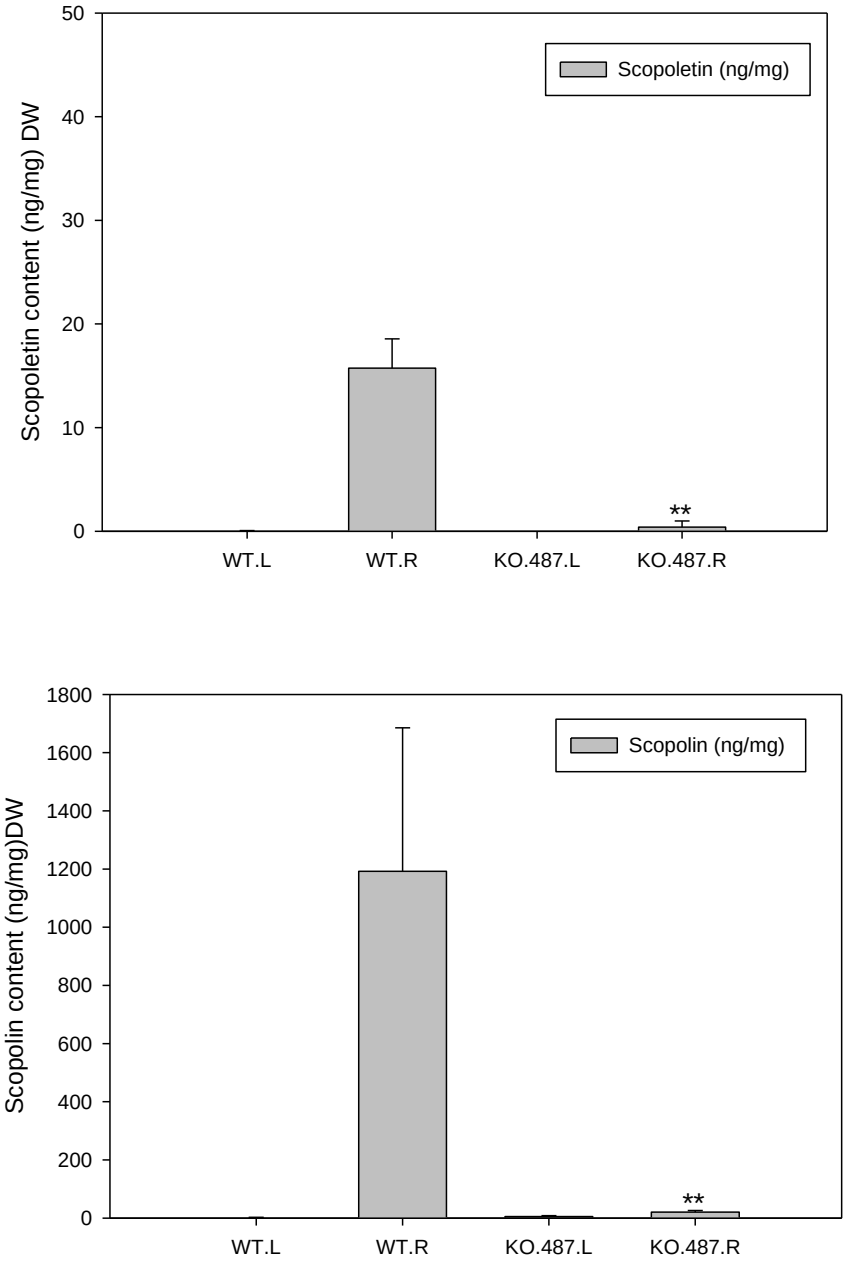


Figure 4.4. Analysis of scopoletin and scopolin contents in sample wild type and scopoletin-GT knock out lines, SALK_097487 using LC-MS. WT: wild type, 487: SALK_097487, KO: knock out, L: Leaf, R: Root, (**): highly statistically significant different (p value < 0.01) by student T-test.

There was a statistically significant reduction in scopolin content in root samples of scopoletin-GT knock out lines, SALK_097487 (20.69 ± 5.27 ng/mg DW), which was approximately 57-fold lower than that of the wild type (1192.06 ± 493.26 ng/mg DW). Surprisingly, scopoletin in the knock-out mutant also decreased (0.4 ± 0.6 ng/mg DW), which was 39-fold lower than that obtained in wild type (15.74 ± 2.83 ng/mg DW).

Whereas in Arabidopsis scopolin-BG knock out line, GK-732F07, there was no statistically significant different in scopoletin content between knock out mutant (17.37 ± 12.72 ng/mg DW) and wild type (15.74 ± 2.83 ng/mg DW). Scopolin content in knock out mutant also remained at similar levels as wild type, 1,222.93 ± 47.07 ng/mg DW and 1,192.06 ± 493.26 ng/mg DW, respectively (Figure 4.5).

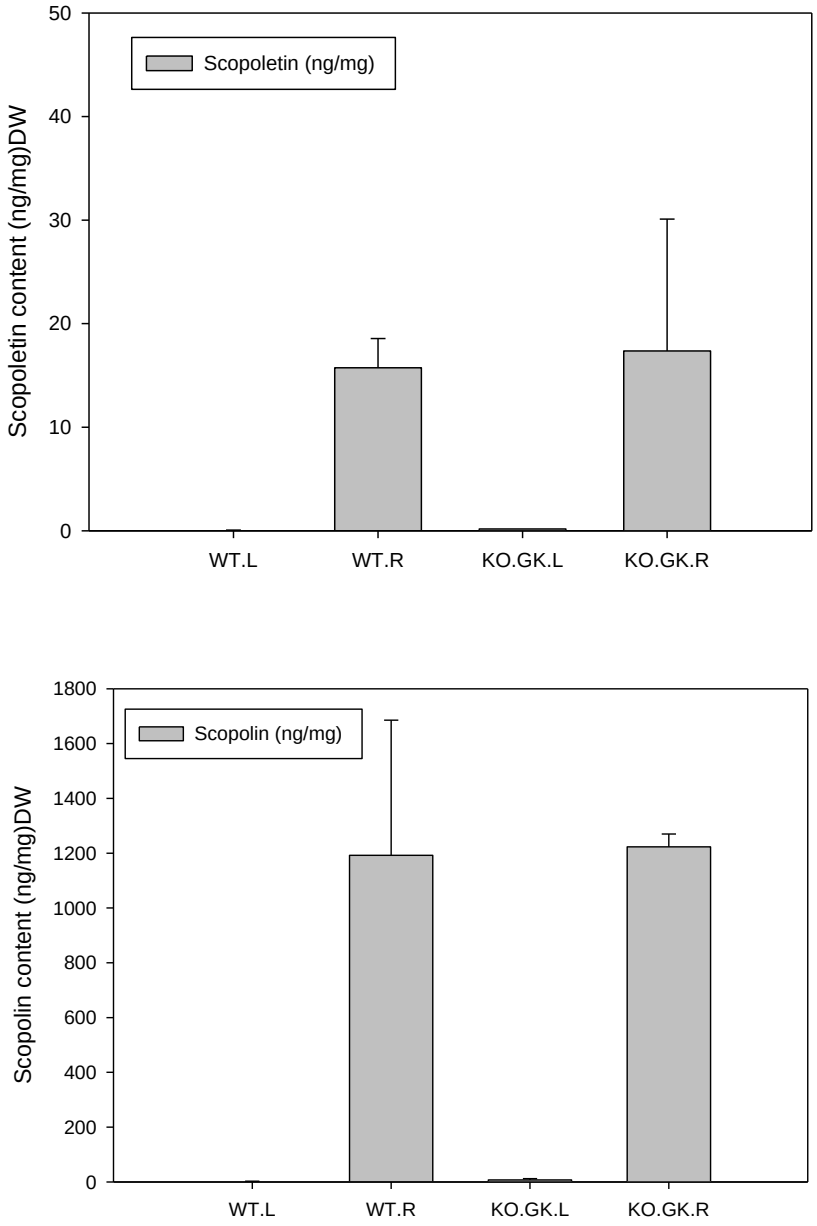


Figure 4.5. Analysis of scopoletin and scopolin contents in sample wild type and scopolin-BG knock out line, GK-732F07 using LC-MS. WT: wild type, GK: GK-732F07, KO: knock out, L-Leaf, R-Root.

4.3.2. Creation of overexpression constructs via GATEWAY cloning technology

4.3.2.1. Full-length cDNA amplification of candidate cassava genes

Samples of cDNA were prepared from cassava root, and full-length cDNAs of candidate genes: *MeSGT*1 for scopoletin-GT and *MeSBG*4 for scopolin-BG were amplified by PCR using gene specific primers that were designed within 5'-untranslated regions (5'-UTR) and 3'-UTR (section 2.5.3). A high fidelity Q5® DNA polymerase was used to minimise mutations during cDNA amplification. PCR products of full-length cDNA were checked on gel electrophoresis and were visualised under the UV light (Figure 4.6).

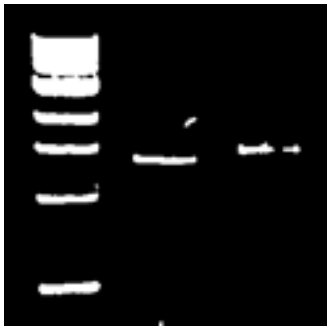


Figure 4.6. Amplification of full-length cDNA of *MeSGT*1-1427bp (A) and *MeSBG*4-1526bp (B) by PCR using High fidelity Q5® DNA polymerase.

4.3.2.2. Producing attB-flanked PCR product and T/A cloning

In order to suit the GATEWAY cloning, two recombination sites, *att*B1 and *att*B2 were added to the PCR products of full-length cDNA by PCR reaction with *att*B primers and Q5® DNA polymerase. The PCR products generated by Q5® DNA polymerase have blunt ends, which is not suitable for cloning such as T/A cloning. Therefore, the products were purified and were added A-overhangs using Taq DNA polymerase. The full-length cDNA containing *att*B sites and A-overhangs at both 5'- and 3'-end, further called insert were then cloned into TA vector, pGEM® T-Easy vector system I via T/A cloning (Figure 4.7).

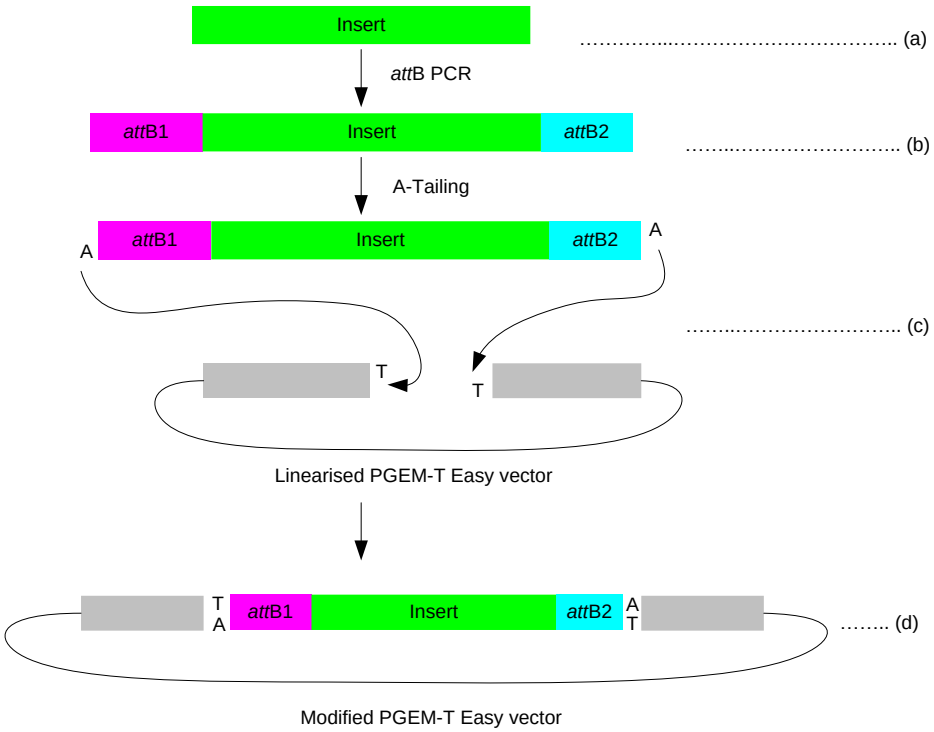


Figure 4.7. Producing *att*B flanked insert vector via T/A cloning. a) Insert (full-length cDNA), b) *att*B PCR, c) TA-cloning, d) Modified pGEM®-T-Easy vector containing the insert.

Modified pGEM® T-Easy vector containing insert were transferred into 10-beta competent *E. coli* via heat shock. The transformants *E. coli* were screened on LB plate containing Ampicillin 100 mg/L supplemented with XGAL 1 mg/mL and IPTG 2.4 mg/mL. The presence of the insert in the selected transformants was checked by colony PCR and its sequence was confirmed by DNA sequencing using general M13 primer sequencing. The pGEM® T-Easy vector containing correct *att*B-flanked insert was cloned into the expression vector, pCAMBIA 1305.1 using the GATEWAY cloning.

4.3.2.3. The GATEWAY Cloning Technology

This method consists of two recombination reactions: BP recombination reaction to create an entry clone and LR recombination reaction to create expression clone.

BP recombination reaction

The pGEM® T-Easy vector containing insert were linearised with restriction enzyme *Pst*I1 and were cloned into pDONR/Zeo via BP recombination reaction (Figure 4.8). The *att*B sites of pGEM® T-Easy vector react specifically with *att*P sites of the pDONR/Zeo resulting in an entry clone for LR recombination reaction.

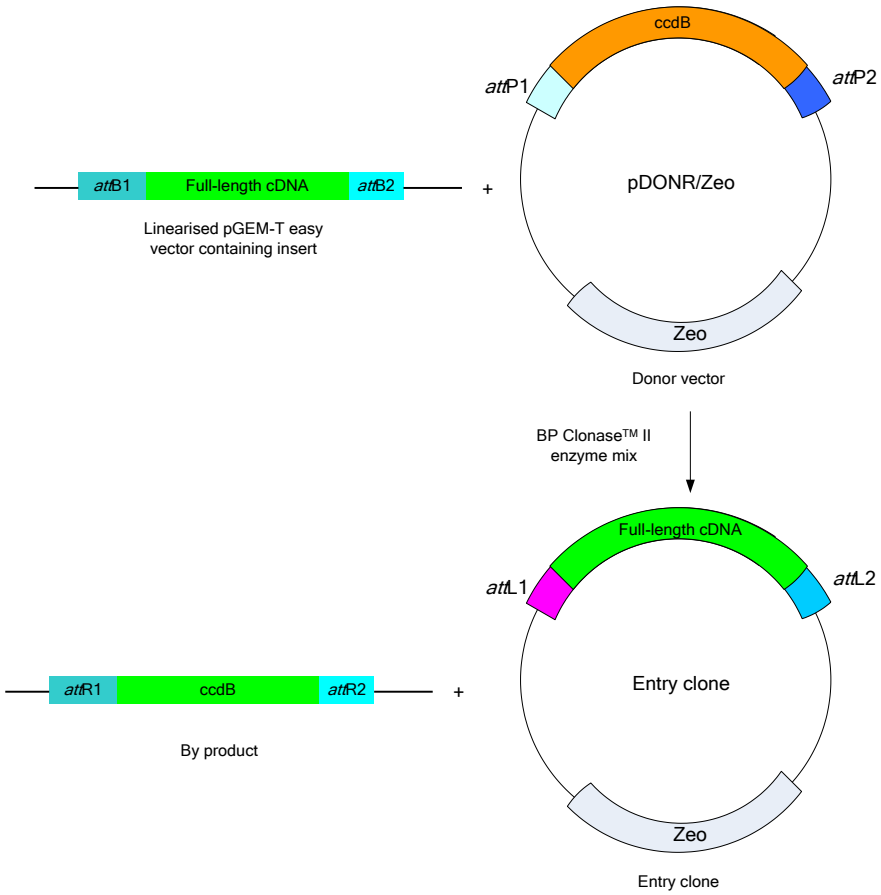


Figure 4.8. Production of an entry clone via BP recombination reaction using linearised pGEM® T-Easy vector and pDONR/Zeo.

LR recombination reaction

The entry clone from BP product was then cloned into the expression vector, modified pCAMBIA 1305.1 via LR reaction (Figure 4.9).

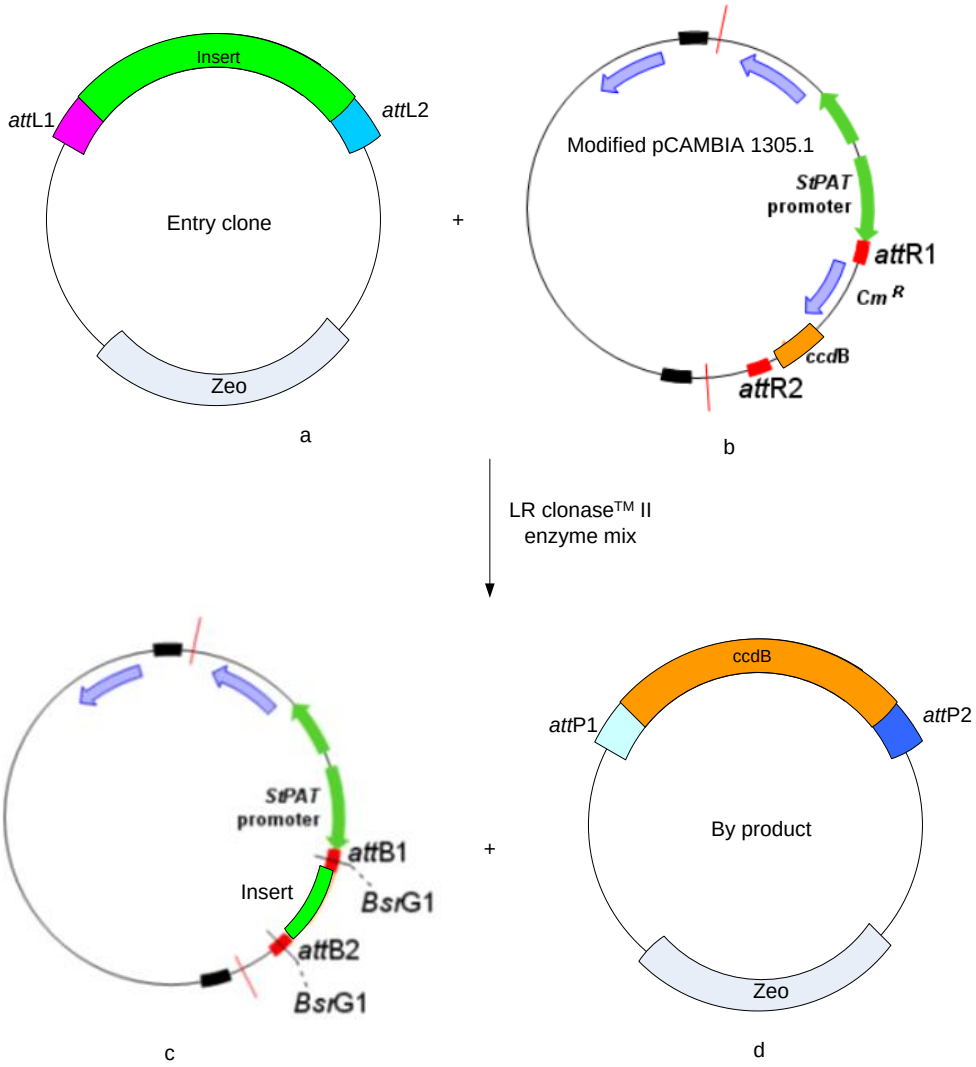


Figure 4.9. Production of expression vector via LR recombination reaction using PDONR/Zeo and modified pCAMBIA 1305.1.

The LR products, pCAMBIA 1305.1::insert, were put in 10-beta competent *E. coli* via heat shock transformation. The presence of the insert in the

transformants was checked using colony PCR and was confirmed using DNA sequencing. Four gene constructs expressing *MeSGT*1 for scopoletin-GT and *MeSBG*4 for scopolin-BG under the control of different promoters were created (Table 4.3). The gene constructs were put into *Agrobacterium tumefaciens* strain GV3101 using electroporation method and were used in Arabidopsis transformation.

Table 4.3. *MeSGT*1- and *MeSBG*4-overexpressing constructs for Arabidopsis transformation.

No	Cassava gene	Promoter
1	*MeSGT*1 encoding scopoletin-GT	CaMV 35S
2	*MeSGT*1 encoding scopoletin-GT	*StPATATIN*
3	*MeSBG*4 encoding scopolin-BG	CaMV 35S
4	*MeSBG*4 encoding scopolin-BG	*StPATATIN*

4.3.3. Production and selection of Arabidopsis transgenic lines

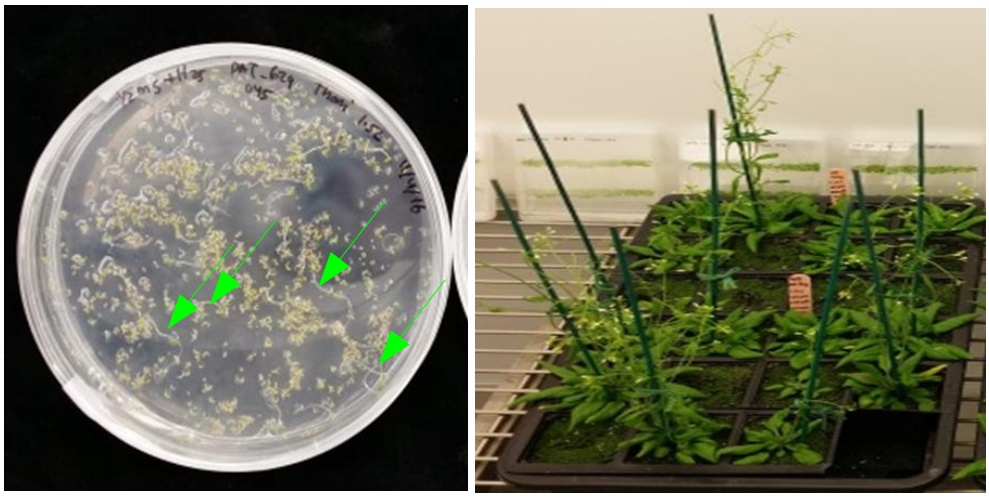
4.3.3.1. Transformation of Arabidopsis using dip floral method

To produce Arabidopsis transgenic lines, 5-week-old plants were transformed with Agrobacterium carrying either *MeSGT*1- or *MeSBG*4-overexpressing constructs using dip floral method (Clough and Bent, 1998). The transformed plants were air dried for 2-3 hours, covered with black plastic overnight and allowed to grow until the seeds were ready for harvest (Figure 4.10).

Figure 4.10. Transformed Arabidopsis plants grown in a growth room under controlled condition of 21-22 ºC, 50-60% relative humidity, and 16 h daylight.

Selection of transgenic plants using Hygromycin-selection medium

Transgenic Arabidopsis were screened on MS agar medium containing 25 mg/L of Hygromycin. After dark-incubation for five days, those with elongated hypocotyl and closed cotyledons were marked as transgenic and transferred to soil for growth (Figure 4.11).



A B

Figure 4.11. Screening of transgenic Arabidopsis on MS agar plate. Hygromycin-resistance seedlings selection on MS agar containing 25 mg/L Hygromycin (A) and growth of transgenic Arabidopsis (B) under controlled condition of 21-22 ºC, 50-60% relative humidity, and 16 h daylight.

Several transgenic lines for each construct were generated (Table 4.4).

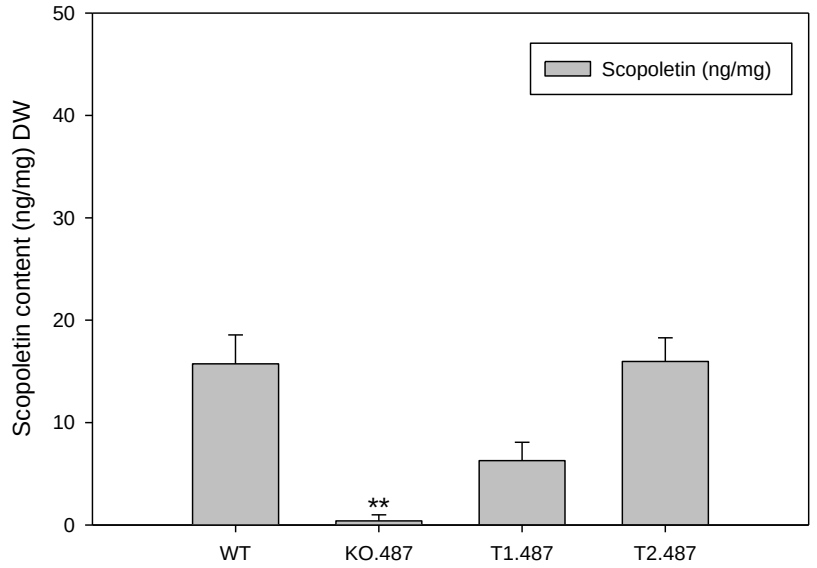
Table 4.4. Variation of hygromycin-resistant seedlings produced from each transformed mutant.

No	Cassava gene candidate	T-DNA insertion line	Promoter	No of hyg-resistant seedlings
1	*MeSGT*1	SALK_097487	CaMV 35S	18
2	*MeSGT*1	SALK_097487	*StPATATIN*	21
3	*MeSBG*4	GK-732F07	CaMV 35S	2
4	*MeSBG*4	GK-732F07	*StPATATIN*	-

Scopolin-BG knock out mutant, GK-732F07, showed low fertility affecting the availability of seed material for the hygromycin-resistant plant selection. Thus, less transgenic plants were obtained (Table 4.4). The seeds from transgenic plants (T_1) were directly used for biochemical analysis to see whether cassava genes complement the mutants.

4.3.4. Functional confirmation of *MeSGT*1 and *MeSBG*4 in transgenic Arabidopsis

In this section, we measured scopoletin and scopolin in root samples only because the previous results (section 4.3.1) have shown that scopoletin and scopolin levels in leaf samples were very low and no significant difference between wild type and knock out lines. The LCMS analysis of two *MeSGT*1 overexpressing-transgenic SALK_097487 lines: T1.487 driven by 35S promoter and T2.487 driven by *StPATATIN* promoter showed that the levels of scopoletin and scopolin increased up to the levels of wild type, particularly in T2.487 line (Figure 4.12). Scopoletin content in roots of T1.487 line and T2.487 line was 6.29 ± 1.79 ng/mg DW and 15.97 ± 2.31 ng/mg DW, respectively. The latter line showed similar levels to that of wild type (15.74 ± 2.83 ng/mg DW).



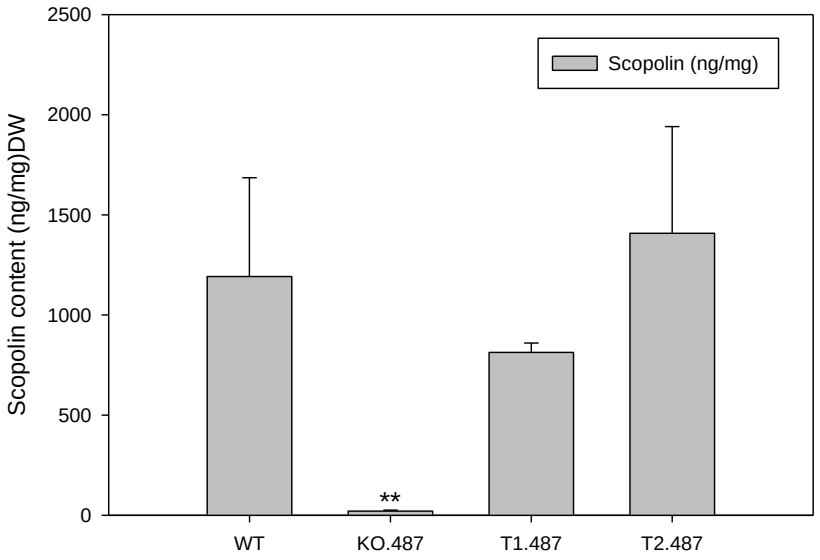


Figure 4.12. Analysis of scopoletin and scopolin contents by LCMS in *MeSGT*1-overexpressing Arabidopsis transgenic line, SALK_097487. WT: wild type, KO: knock out, T1: transgenic line 1::35S promoter, T2: transgenic line 2::*StPATATIN* promoter, 487: SALK_097487.

While in two *MeSBG*4-overexpressing Arabidopsis lines: T1.GK line and

T2.GK line, there was no significant changes in scopoletin and scopolin accumulation. Scopoletin levels in *MeSBG*4-overexpressing Arabidopsis T2.GK line was 11.57 ± 8.3 ng/mg DW, which was slightly lower compared to the wild type of 15.74 ± 2.83 ng/mg DW. While scopolin content in T2.GK line was 1,098.73 ± 913.69 ng/mg DW and 1,192.06. ± 493.26 ng/mg DW in the wild type (Figure 4.13).

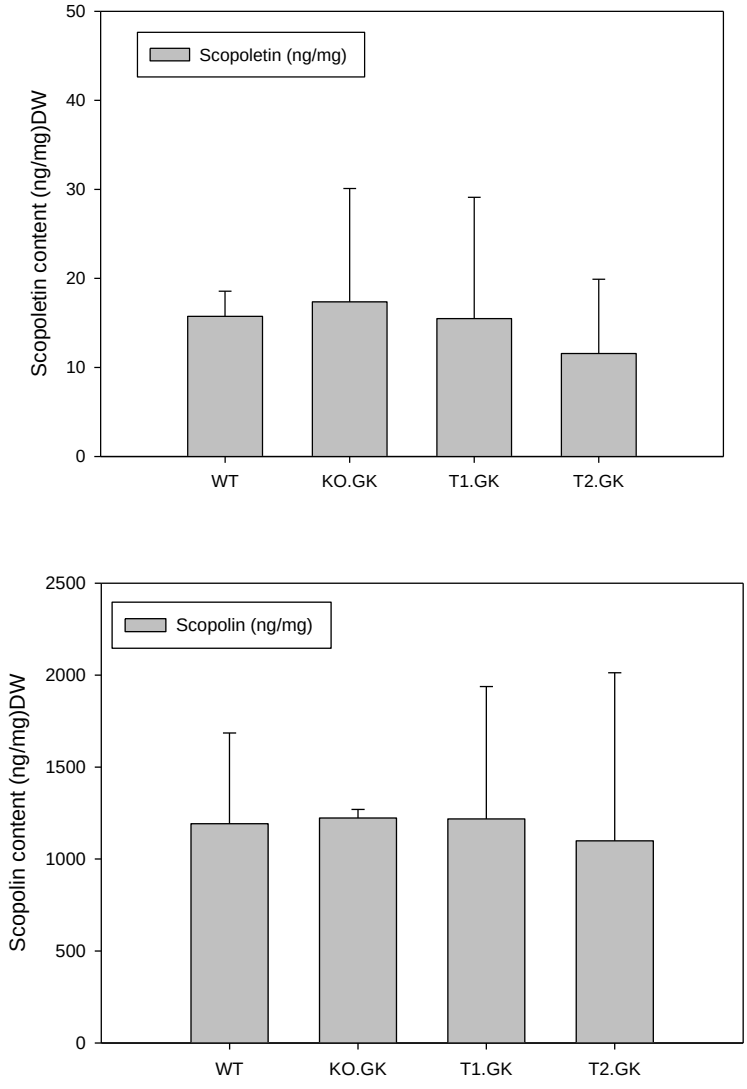


Figure 4.13. Analysis of scopoletin and scopolin contents in *MeSBG*4-overexpressing Arabidopsis transgenic line, GK-732F07. WT: wild type, KO: knock out, T1: transgenic line 1::35S promoter, T2: transgenic line 2::35S promoter.

4.4. Discussion

This chapter aimed to confirm the function of the selected cassava genes for scopoletin-GT and scopolin-BG using Arabidopsis T-DNA knock out lines. This work is crucial in order to select the correct candidate gene prior to the genetic modification. Our results confirm that cassava scopoletin-GT, *MeSGT*1, complement Arabidopsis mutant, while cassava scopolin-BG, *MeSBG*4, remains unclear.

We found that knocking out the gene encoding scopoletin-GT, At4g34131, in Arabidopsis reduced both scopoletin and scopolin content significantly in the mutant, SALK_097487. The decreased scopoletin was unexpected. However, the same finding was shown in scopoletin-GT down-regulated transgenic tobacco (Chong et al., 2002). It suggests that scopoletin cannot accumulate to high levels in the plant cells, which might be due to its potential toxicity in free form and it is subject to oxidation by cellular peroxidase (Chong et al., 2002). Therefore, compromising scopoletin accumulation from de-glucosylation of scopolin might enhance the activity of cellular peroxidase, leading to the increased oxidation of scopoletin. Under normal conditions, plants have their own mechanism to protect the cells from chemical reactivity or toxicity of free form phenylpropanoids by converting them to its glucoside via glucosylation by the action of UDP-Glc:glucosyltransferases (UGTs) (Vogt and Jones, 2000, Li et al., 2001). This means that UDP-glucosyltransferases is activated in the metabolic pathway at all times during normal cellular processes although they are also induced by stress conditions such as wounding in cassava (Reilly et al., 2007, Owiti et al., 2011). The glucosylation enhances the water solubility of secondary metabolites so that they can be stored in vacuole (Li et al., 2001). In addition, glucosylation plays an important role in plant defense because phenylpropanoid glucosides such as glucosylated scopoletin, scopolin, are also known to have toxic effect towards *Sclerotinia sclerotiorum* (Prats et al., 2006).

This study reveals the function of the selected cassava scopoletin-GT, *MeSGT*1. Quantification by LCMS in *MeSGT*1-expressing Arabidopsis transgenic line showed scopolin levels in knock out line was returning back at similar levels to that of wild type, confirming that *MeSGT*1 possess UDP-glucosyltransferase towards scopoletin substrate. Interestingly, the levels of scopoletin in transgenic Arabidopsis was also back to the levels of wild type, which suggests that glucosylation step plays important role in the homeostasis of the phenylpropanoid (Le Roy et al., 2016). Furthermore, it is interesting to see whether knocking down *MeSGT*1 in cassava would have the same effects on scopoletin and scopolin content as shown in transgenic tobacco and Arabidopsis. Down-regulation of *MeSGT*1 in cassava and its effect on cassava PPD response will be discussed in chapter 6.

On the other hand, knocking out the BGLU23 gene for scopolin beta-glucosidase (scopolin-BG) in Arabidopsis did not show significant changes in scopoletin and scopolin contents. It was expected that knocking out the scopolin-BG would inhibit the conversion of scopolin to scopoletin, thereby resulting in the increase of scopolin accumulation in the cells. We assume that this unexpected result might be due to the role of the gene in the biosynthetic pathway, which is more likely to be activated in certain conditions such as under abiotic and biotic stresses to release scopoletin as a plant defense compound. Therefore, down regulating or knocking out the gene for scopolin-BG in this pathway did not show a significant impact on the scopoletin and scopolin contents in fresh tissue. To confirm this hypothesis, more studies are required to study the gene expression profiles in fresh tissue and under stress conditions such as wounded or infected tissues, which can be done using fresh and wounded cassava roots.

Furthermore, the function of the selected cassava scopolin-BG, *MeSBG*4, remains unclear. Because there were no significant changes in scopoletin

and scopolin between wild type and knock out mutant, we could not determine the function of *MeSBG*4 in Arabidopsis. Moreover, biochemical analysis results of *MeSBG*4-expressing-transgenic Arabidopsis lines also showed no significant different in the accumulation of scopoletin and scopolin. This leads to another hypothesis that the homologous cassava gene may not possess specific activity towards scopolin or its specificity is low. The previous results in chapter 3 have shown that this gene shared low similarity in their amino acid sequence and the best candidate cassava gene for this enzyme that most closely related to the reference gene, BGLU23 could not be identified. Therefore, we alternatively overexpressed BGLU23 gene in cassava to alter the interconversion of scopoletin and scopolin pathway in cassava and study their effect on the scopoletin and scopolin accumulation in cassava that may affect PPD response. The overexpression of BGLU23 in cassava and its effect on cassava PPD response will be discussed in chapter 7.

In addition, we found that the nature of scopoletin and scopolin accumulation in the fresh Arabidopsis roots are different compared to other plants such as tobacco and cassava. In Arabidopsis roots, both scopoletin and scopolin levels are noticeably high compared to those two plants. Particularly for scopoletin, its accumulation was considerably high about 15 ng/mg DW, while in other plants it is usually present at trace amounts. In addition, scopolin accumulation in Arabidopsis roots was also significantly higher than that of tobacco or cassava (Chong et al., 2002, Gachon et al., 2004, Tanaka et al., 1983, Buschmann et al., 2000, Bayoumi et al., 2010).

Chapter 5. Generating cassava scopoletin glucosyl-transferase, *MeSGT*1, RNAi- and Arabidopsis scopolin beta-glucosidase, BGLU23, -expressing transgenic cassava

Chapter 5. Generating cassava scopoletin glucosyl-transferase, *MeSGT*1, RNAi- and Arabidopsis scopolin beta-glucosidase, BGLU23, -expressing transgenic cassava

5.1. Introduction

The aim of this study was to investigate the role of the interconversion of scopoletin and scopolin in cassava PPD by inhibiting the expression of cassava scopoletin-glucosyltransferase (scopoletin-GT), *MeSGT*1, via RNAi construct and by overexpressing Arabidopsis scopolin-beta-glucosidase (scopolin-BG), BGLU23. The latter approach was alternatively chosen because we were not able to identify a strong candidate cassava gene encoding scopolin-BG for RNAi gene silencing (chapter 3). These two approaches should have the potential to disrupt the interconversion of scopoletin and scopolin in cassava storage roots, which could alter scopoletin and scopolin levels in the roots, thereby affecting PPD response in transgenic cassava. In order to generate transgenic cassava, we used *MeSGT*1-RNAi and BGLU23-overexpression constructs to transform friable embryogenic callus (FEC) of the West African Cassava cultivar (cv) 60444 using *Agrobacterium tumefaciens* strain LBA4404. The use cv 60444 as a model because it has good regeneration capacity of embryogenic tissue. Agrobacterium-mediated cassava FEC transformation has been widely used to produce transgenic cassava (Zainuddin et al., 2012, Bull et al., 2009, Gonzalez et al., 1998). However, the cassava transformation is complex and can be challenging; therefore, it was carried out in collaboration with the cassava research group at ETH, Zurich.

5.2. Aims of study and research strategies

The aim of this chapter was to generate transgenic cassava expressing *MeSGT*1-RNAi and full-length cDNA of BGLU23.

In order to achieve the aim of the study, we used strategies as follows:

1. Create *MeSGT*1-RNAi and BGLU23-overexpression constructs via GATEWAY cloning technology.

2. Transform cassava friable embryogenic callus (FEC) cv. 60444, regenerate transgenic lines and identify single-insert copy number lines.

3. Multiply independent single-insert lines for growth under glasshouse conditions in order to harvest sufficient roots for analysis.

5.3. Results

5.3.1. Creation of *MeSGT*1-RNAi and BGLU23-overexpression constructs via GATEWAY cloning technology

Generating hairpin structure for MeSGT1-RNAi constructs

Hairpin structure for RNAi construct was first made from 332 bp fragments of *MeSGT*1 gene sequence that shares 76% similarity with another target gene, *MeSGT*6, (Figure 5.1).

```
Identities = 254/332 (76%),
Positives = 254/332 (76%), Gaps = 0/332 (0%)

MeSGT1 target RNAi region    811 GAGTGTCTGAAATGGCTTGATGCGAAGAAACCCAATTCTGTTCTTAACGTATGCTTTGGT  870
                                 GAGTGT TGAAATGGCT GA  C AA AAACCCAATTCTGT  T  AC TATG TTTGG
MeSGT6 target RNAi region    823 GAGTGTTTGAAATGGCTCGACTCCAACAAACCCAATTCTGTAATCTACATATGTTTTGGA  882

MeSGT1 target RNAi region    871 ACGGTTACAAAGTTTTCTGACTCCCAGCTACATGAGATTGCAATAGGGCTTGAAGCTTCA  930
                                 A  GT  C AA TT   TG CTC CAGCT    GAGATTGC AT G  CTTGAA CTTCA
MeSGT6 target RNAi region    883 AGTGTGGCTAACTTCAGTGCCTCTCAGCTTATGGAGATTGCCATGGCTCTTGAATCTTCA  942

MeSGT1 target RNAi region    931 AAGCAAAATTTCATCTGGGTTGTTAGGAAAGACAAGAACGAAGAAGAATCTGAAGAGAAA  990
                                 AAGCA  A TTCAT TGGGT GT AG A AGACAAGAAC A  AAGAA  TGAAGAGAA
MeSGT6 target RNAi region    943 AAGCAGCAATTCATTTGGGTGGTGAGAAGAGACAAGAACTATAAAGAAGATGAAGAGAAG 1002

MeSGT1 target RNAi region    991 TGGTTGCCTGAGGGGTATGAGAAAAGAATGGAAGGAAAGGGACTAATTATAAGGGGATGG 1050
                                 TGGTT CCTGA GG T TGAG AAAGAATG AAG  AAGGG CT ATTATAAG GGATGG
MeSGT6 target RNAi region   1003 TGGTTACCTGAAGGATTTGAGGAAAGAATGAAAGAGAAGGGCCTCATTATAAGAGGATGG 1062

MeSGT1 target RNAi region   1051 GCACCCCAAATTTTGATTCTTGATCATGAAGCTATTGGTGGGTTTATTACTCACTGTGGG 1110
                                 GCACC CAA T TTGATTCTTGATCATGAAGC ATTGG GG TTT T AC CACTG GG
MeSGT6 target RNAi region   1063 GCACCACAAGTGTTGATTCTTGATCATGAAGCCATTGGAGGATTTGTGACACACTGCGGA 1122

MeSGT1 target RNAi region   1111 TGGAACTCTACTTTAGAAGGTGTGAGTGCCGG 1142
                                 TGGAA TCTAC  T GAAGG  T A TGC GG
MeSGT6 target RNAi region   1123 TGGAATTCTACACTTGAAGGCATAACTGCTGG 1154
```

Figure 5.1. The alignment of nucleotide sequences of RNAi target region. Length of sequence: 332bp, Identical site: 254, Pairwise % Identity: 76%, GC content: 42.3%, Alignment type: Global alignment with free end gaps, cost matrix: Blosum 62, Gap open penalty: 12, Gap extension penalty: 3, by Geneious software.

The PCR fragments carrying two recombination sites (*att*B1 and *att*B2)

suitable for GATEWAY cloning and appropriate restriction sites for ligation (Figure 5.2) were used to create sense and antisense fragments. The PCR products were checked on gel electrophoresis and were visualised under the UV transilluminator (Figure 5.3).

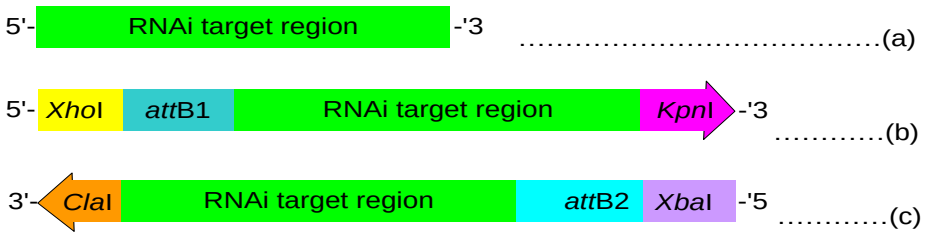


Figure 5.2. Producing *att*B-flanked PCR product for RNAi vector. (a). Target RNAi region (332 bp); (b). Sense fragment containing *att*B1 and appropriate restriction sites (*Xho*I and *Kpn*I); (c). Antisense fragment containing *att*B2 and appropriate restriction enzymes (*Xba*I and *Cla*I).

Figure 5.3. Gel electrophoresis of sense and anti-sense fragments. S: sense fragment, S1: 332 bp fragment of *MeSGT*1 gene, S2: 12*att*B PCR product containing *Kpn*I at 3' end, S3: full *att*B1 PCR product, S4: sense fragment with *Xho*I at 5' end and *Kpn*I at 3' end suitable for ligation to create hairpin structure in pKANNIBAL vector (Figure 5.2b). AS: antisense fragment, AS1: 332 bp fragment of *MeSGT*1 gene, S2: 12*att*B PCR product containing *Cla*I at 3' end, S3: full *att*B1 PCR product, S4: sense fragment with *Xba*I at 5' end and *Cla*I at 3' end suitable for ligation to create hairpin structure in pKANNIBAL vector (Figure 5.2c).

The sense and antisense fragments were then cloned into pKANNIBAL vector to create a hairpin RNA structure. First, the sense fragment was inserted into the pKANNIBAL vector via restriction digestion and ligation (Figure 5.4).

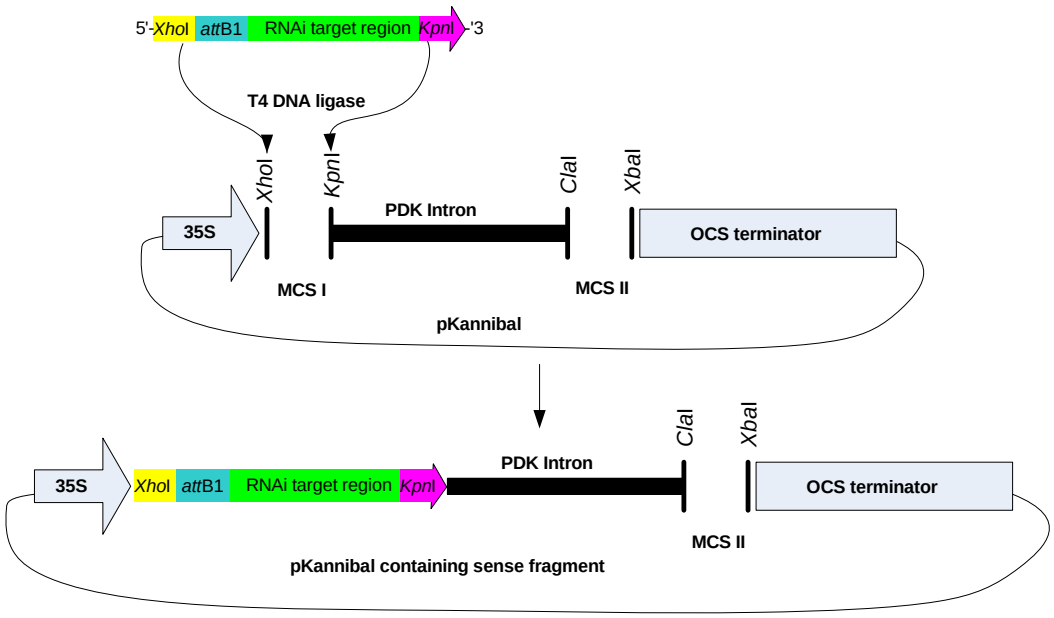


Figure 5.4. Cloning of sense fragment containing *Xho*I and *att*B1 at 5'-end and *Kpn*I at 3'-end into the pKANNIBAL vector.

The cloning product of pKANNIBAL containing sense fragment was put in 10-beta competent *E. coli* via heat shock transformation. The presence of the sense fragment in the transformants *E. coli* was checked by colony PCR with gene specific primers (Figure 5.5) and its sequence was confirmed by DNA sequencing with specific primers sequencing based on promoter and PDK intron regions in the pKANNIBAL vector.

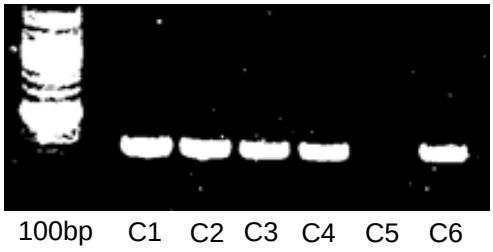


Figure 5.5. Detection of *MeSGT*1-sense fragment in the transformants *E. coli*::pKANNIBAL by Colony PCR. C: colony, 1-6: colony number. The insert was not identified in C5. C2, C3, and C6 were selected for DNA sequencing.

After confirming the sense fragment in the pKANNIBAL, antisense fragment (Figure 5.2.c) was then cloned into the pKANNIBAL::*MeSGT*1-sense fragment via restriction digestion and ligation (Figure 5.6) to complete the hairpin structure. The presence of antisense fragment in the vector was checked by colony PCR (Figure 5.7) and its sequence was confirmed by DNA sequencing with primers designed based on PDK intron and terminator sequence.

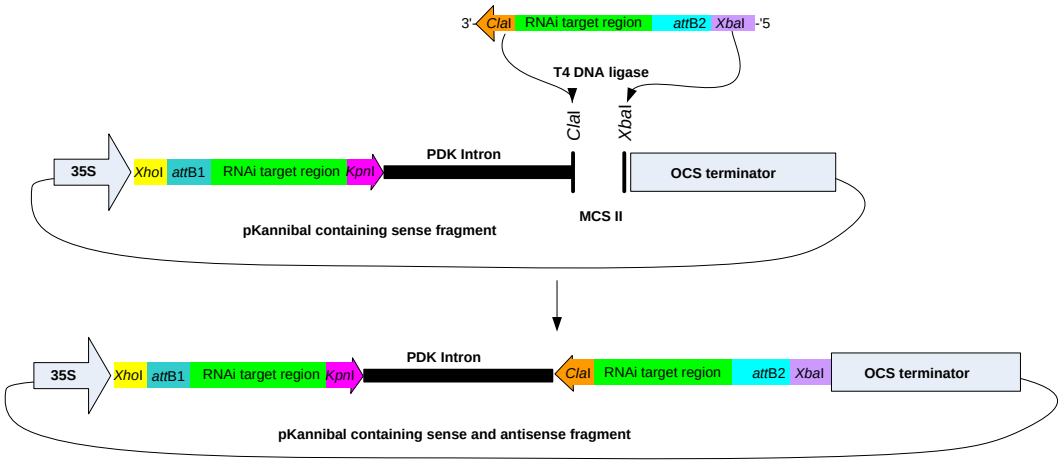


Figure 5.6. Cloning of antisense fragment into the pKANNIBAL::*MeSGT*1-sense fragment to create hairpin structure.

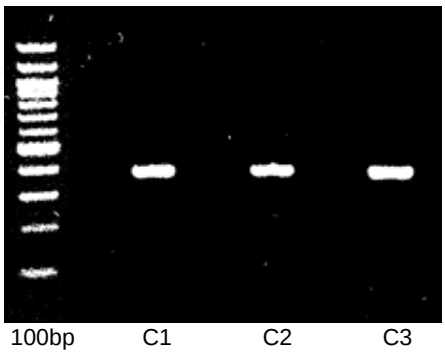


Figure 5.7. Detection of *MeSGT*1-antisense fragment in the transformants *E. coli* by Colony PCR. C1-C3: Colony no 1-3.

The hairpin structure with sense and antisense fragment of *MeSGT*1 gene in the pKANNIBAL vector (Figure 5.6) was then cloned into the expression vector, pCAMBIA 1305.1 via GATEWAY cloning involving BP and LR recombination reactions, which will be discussed in the next section.

Amplification of full-length cDNA of BGLU23 for overexpression constructs

Full-length cDNA of scopolin-BG from Arabidopsis, BGLU23 (1575 bp) prepared from roots was amplified by PCR with high fidelity Q5 DNA polymerase. Two recombination sites (*att*B1 and *att*B2) suitable for GATEWAY cloning were added at the both 5' and 3'-end of the resulting product by PCR with *att*B primers. The *att*B PCR product, further called insert was then cloned into TA vector, PGEM T-Easy system I with the addition of A-overhangs prior to the cloning (Figure 5.8). All PCR products were confirmed on gel electrophoresis (Figure 5.9).

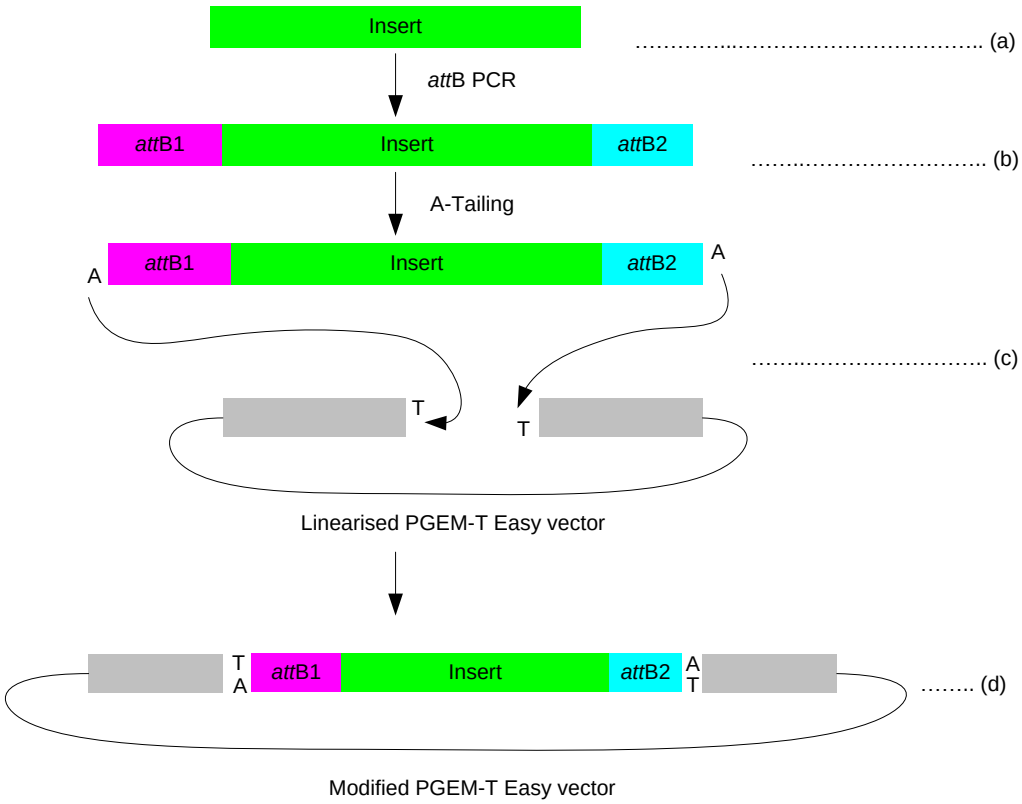


Figure 5.8. Producing attB flanked insert vector. a. Insert (full-length cDNA of BGLU23), b. *att*B PCR product, c. TA-cloning, d. The pGEM®-T Easy vector containing the insert.

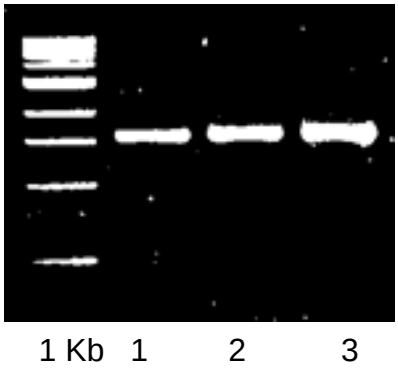


Figure 5.9. Gel electrophoresis of full-length cDNA of BGLU23 (1), 12 *att*B (2) and full *att*B fragments (3) generated by PCR with specific gene primers and high fidelity Q5 DNA polymerase.

The pGEM®-T Easy vector containing the insert was used to transform 10-beta competent *E. coli* and the transformants were selected on agar selection medium containing Ampicillin 100 mg/L. The presence of the insert was checked by colony PCR with gene specific primers (Figure 5.10) and its sequence was confirmed by DNA sequencing with general M13 primer sequencing. The pGEM® T-Easy vector with correct insert sequence was firstly linearised with *Pst*I before it was cloned into the pCAMBIA 1305.1 via GATEWAY cloning.

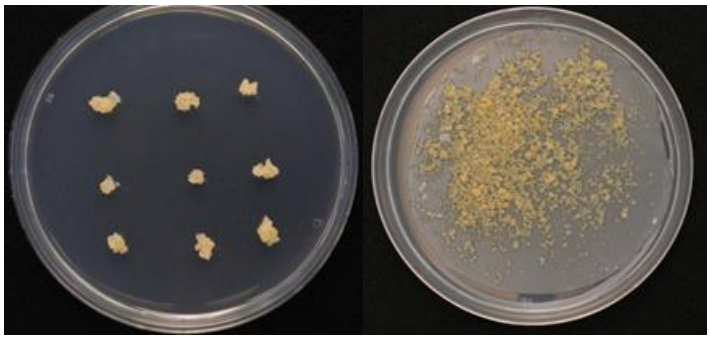
Figure 5.10. Analysis of full-length cDNA of BGLU23 in the transformants *E. coli*::pGEM® T-Easy vector by colony PCR. C: colony, 1-8: colony number. The insert was not identified in C1 only and C2-C8 were selected for DNA sequencing.

GATEWAY cloning technology to produce an expression vector for cassava transformation

Both the pKANNIBAL::*MeSGT*1-sense and antisense fragments and pGEM® T-Easy::BGLU23 were cloned into the pCAMBIA 1305.1 driven by either constitutive CaMV-35S or root-specific *StPATATIN* promoters using GATEWAY cloning (section 2.10.5.7, Figure 4.8 and Figure 4.9). A total of four constructs including two *MeSGT*1-RNAi and two BGLU23-overexpression constructs were created. In addition, GUS Plus reporter gene-expressing constructs was used as a control to evaluate the transformation success. The constructs were put in *A. tumefaciens* strain LBA4404 via electroporation and were used for cassava transformation.

5.3.2. Transformation of cassava friable embryogenic callus (FEC) at ETH Zurich

About 2-week-old FECs on GD medium were transformed with Agrobacterium carrying either *MeSGT*1-RNAi or BGLU23-overexpression constructs and were transferred onto a sterile 100 μm nylon mesh for the maturation of FEC and regeneration steps (Figure 5.11). After the transformation was complete, the transformed FECs were transported to University of Bath for the maturation, regeneration and analysis.

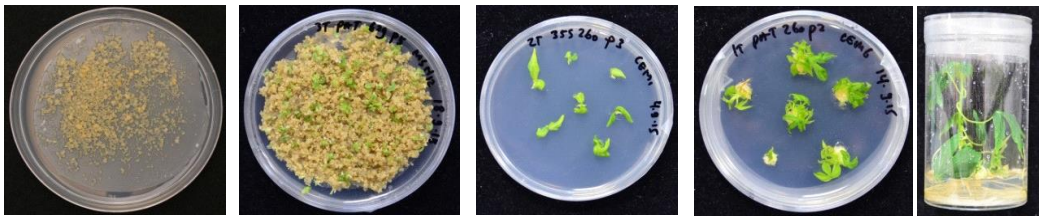


A B

Figure 5.11. Agrobacterium-mediated transformation of cassava FEC. A. Starting material, FECs on GD plate, B. Transformed FECs on selection medium, GD plate supplemented with carbenicillin 250 mg/L. The transformed FECs were put on 100 μm nylon mesh to ease the weekly transfer in the regeneration step and were cultured 25-28 ºC, 43-70% relative humidity, 16 h light.

5.3.3. Regeneration, selection and growth of transgenic cassava at University of Bath

The regeneration of transgenic cassava from FEC, which required intensive care for about 8 months, was done successfully at University of Bath (Figure 5.12).



a b c d e

Figure 5.12. Regeneration of transgenic cassava from FECs in the growth room at 28 ºC, 30-50% relative humidity, and 16 h light. a. Transformed FECs on GD+C250 plate, b. Developing cotyledons on MSN+C250+H15 plate after 12 weeks of transfer, c. Selected cotyledons on CEM+C100, d. Selected cotyledons on CEM+C100 after 6 weeks and ready for transfer into CBM medium, e. Growth of plantlet in CBM+C50.

The number of plantlets generated from the transformed FECs was around 70-100 plantlets from each plate indicating that the regeneration capacity of the FECs was good. Several independent single insert lines for both *MeSGT*1-RNAi and BGLU23-overexpression constructs have been generated and been confirmed through various test and analysis as described in the following sections.

GUS expression in transformed FECs and transgenic cassava

To test the transformation success, we performed histochemical GUS assays on both transformed FECs and transgenic cassava with GUS Plus reporter gene-expressing transgenic lines as a control. In this assay, beta-Glucuronidase encoded by the GUS gene cleaves a glucuronic acid bond in X-Gluc structure and produces Chloro-Bromo-Indigo. The oxidation of the latter compound generates an insoluble blue precipitate dichloro-dibromo Indigo (Karcher, 2002). Therefore, tissues carrying the GUS reporter gene turn blue which can be used as a control. GUS assay results showed that the transformation was qualitatively successful (Figure 5.13). The plantlets generated from the transformed FECs were transplanted in CBM medium containing Hygromycin for rooting test to screen putative transgenic plants.

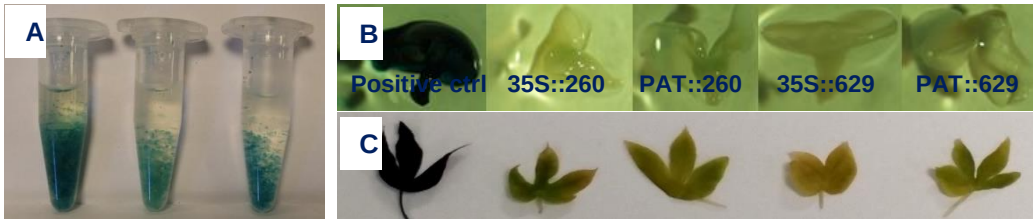


Figure 5.13. Histochemical Gus assay results. A. Control transformed FEC, B. Developing cotyledons on CEM+C100 plate, C. Cassava leaves from transgenic plants. Strong GUS gene expression (blue stained) showed in control and no GUS expression in transgenic samples as expected.

Preliminary screening of transgenic cassava by rooting test

The rooting test is a quick and reliable method to preliminary screen transgenic lines (Bull et al., 2009, Zhang et al., 2000). The plantlets that produced adventitious roots in CBM selection medium containing Hygromycin were considered as Hygromycin-resistance lines or transgenic. After 2 weeks of incubation, most of the tested plantlets produced roots and some did not (Figure 5.14). However, the presence of the insert in the putative transgenic lines obtained from this test still needed to be confirmed by PCR.

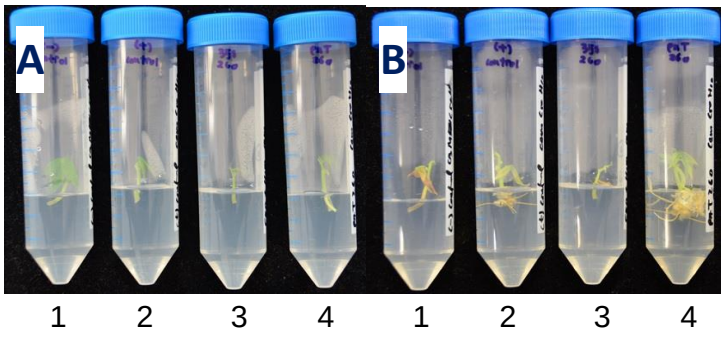


Figure 5.14. Rooting test of cassava transgenic lines and wild-type TMS60444 on CBM+C50+H10 (negative control). A: Week 0, B: Week 2. A1: negative control, untransformed wild type cassava cv. 60444, A2: positive control, wild type cassava cv. 60444 carrying GUS gene, A3: 35S::BGLU23-expressing line, A4: PATATIN::BGLU23-expressing line.

Confirmation of insert in transgenic cassava

The presence of the insert in the putative transgenic lines obtained from the rooting test was confirmed by PCR. Genomic DNA was isolated from leaf samples of the putative transgenic lines and used in the PCR reaction with specific primers that amplified fragments in the Hygromycin region within the transgene (Figure 5.15).

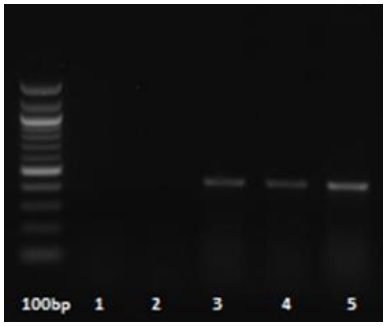


Figure 5.15. Confirmation of the insert by PCR in the putative cassava transgenic lines. The putative cassava transgenic lines (3-5) and wild-type cv. 60444 as negative control (1-2).

Efficiency of cassava transformation

The efficiency of the transformation was calculated based on the rooting test and PCR confirmation results. The results revealed that the efficiency of the transformation was high ranging from 70-100%, which means that most of tested plantlets generated from the FECs were transgenic. Only those with the insert confirmed by PCR were used for southern blotting to determine insert copy number.

Determination of insert copy number in transgenic cassava

Southern blotting enabled us to identify copy number of the insert in the transgenic lines, and determine an independent line (Figure 5.16). In here, we identified not only single insert, but also double and multiple insert from the transgenic lines (Figure 5.16). Those with a single insert only were used for further analysis in this study.

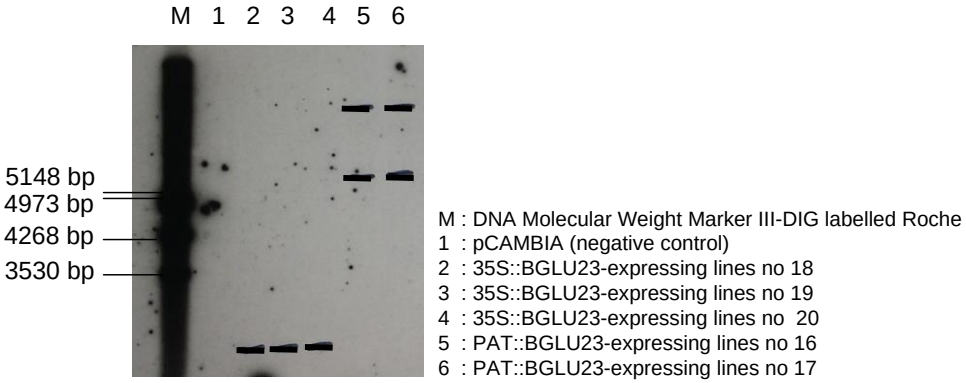
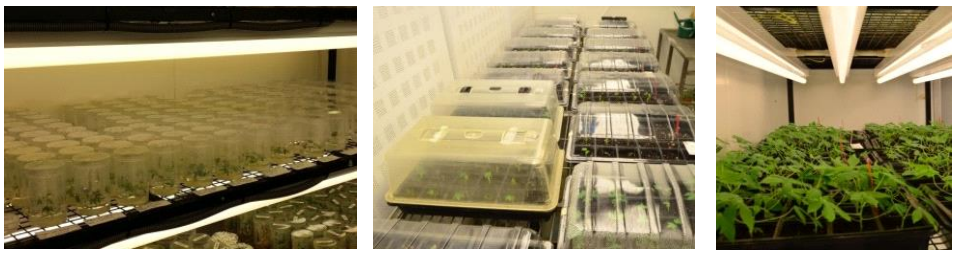


Figure 5.16. Southern blot results of *Hin*dIII-digested cassava genomic DNA of BGLU23-expressing transgenic lines using hygromycin probe. It revealed the number of insert copy number in the transgenic lines. Lane 2-4 show single insert, whilst lane 5-6 show double insert.

5.3.4. *In vitro* propagation and acclimatization of transgenic cassava

Before growing the plants in the glasshouse, selected independent single insert transgenic cassava lines were propagated in CBM agar and were transferred to soil (M2 medium+perlite) after 8-10 days of propagation (Figure 5.17).



a b c

Figure 5.17. In vitro propagation and acclimatization of transgenic cassava. a. Propagation of independent single insert in CBM agar (2.5 g/L), b. Transfer of cassava plantlets in soil, c. Growth of cassava plantlets in soil under controlled condition (25-28 ºC, 30-50% RH and 16 h light).

Growth of transgenic and non-transgenic cassava in a glasshouse

The plantlets were then transferred to a 1-l pot and were grown in the

glasshouse (25-28 ºC, 30-50% RH and 16 h light). To obtain sufficient material for analysis, we grew 30-35 plants for each independent transgenic line with a total line of 16 independent lines including WT (2), *MeSGT*1-RNAi lines (8) and BGLU23-overexpressing lines (6). The plants were watered every day with a great care (Figure 5.18).

a b c

Figure 5.18. Growth of cassava plants in a glasshouse. a. Cassava plantlets in trays from growth room, b. About 2-month-old cassava plants, and c. About 6-month-old cassava plants.

5.4. Discussion

Transformation of cassava FEC via Agrobacterium has been widely used and shown to be the most efficient method to produce transgenic cassava (Gonzalez et al., 1998). However, this method remains a tedious and labour-intensive procedure, which took one year to regenerate plantlets from FECs and get them ready into the glasshouse. In addition, low regeneration of plantlets from somatic embryos (Baba et al., 2008) and highly variable numbers of transgenic events (Koehorst-van Putten et al., 2012) are two main obstacles in the transformation of cassava FEC. To anticipate low regeneration capacity of FECs, the transformation was repeated three times to obtain more FECs for the regeneration step. We also need to consider the potential problem of having either fungal or bacterial contamination that can cause failure in regenerating plantlets from the FECs. Our results showed that the regeneration capacity of the FECs used in this study was good, from which we could generate 70-100 plantlets per plate. The efficiency of the transformation was also relatively high (70-

100%), indicating that the transformation protocols developed by research group at ETH (Bull et al., 2009, Zainuddin et al., 2012) were reliable and reproducible. Southern blotting analysed the numbers of transgenic events in the samples. In agreement with Koehorst-van Putten et al (2012), we identified high variable numbers of transgenic events, which is another difficulty in the transformation of cassava FEC experiment to obtain an independent single insert line.

In this study, we only chose transgenic lines with single copy insert. In the acclimatization step, we found that transferring plantlets at appropriate growth stage when the roots were about 3-5 mm long or about 8-10 days after sub-culturing in agar and applying high humidity in the first 6 days after the transfer to soil critical. Following the original protocol from Bull et al (2009) using 2-week-old plantlets obtained lower percentage of plantlets survival rate because they had been developing long roots that need to be trimmed off before re-planting them into soil, thereby affecting their adaptation to the new environment, from agar to soil. However, this will depend on the growth rate of the plantlets or transgenic lines because the genetic modification that has been made may or may not be affecting the plant growth and developments. Therefore, we would recommend to observe the growth of the plantlets before the acclimatization step as it can vary between lines. A total of 16 independent lines generated in this study were grown in the glasshouse and their roots were harvested after 6 months for various analysis including PPD assessment, biochemical and relative quantification of gene expression analysis, which will be discussed in the chapter 6 and 7. Moreover, while generating transgenic cassava, we also tried performing cassava transformation at University of Bath and it worked well. Our group has recently applied this protocol in the lab with some modifications and generated transgenic cassava successfully.

Chapter 6. Down-regulation of cassava scopoletin-glucosyltransferase reduces scopoletin and scopolin content and delays PPD in cassava

Chapter 6. Down-regulation of cassava scopoletin-glucosyltransferase reduces scopoletin and scopolin content and delays PPD in cassava

6.1. Introduction

Scopoletin plays a central role in cassava postharvest root discoloration or PPD (Liu et al., 2017, Buschmann et al., 2000, Miller et al., 1975, Wheatley and Schwabe, 1985). It accumulates rapidly after injury or microbial attack through *de novo* synthesis from general phenylpropanoid metabolism and is also suggested to be accumulated from the conversion of its glucoside, scopolin (Chong et al., 2002). In cassava, scopoletin is produced from three possible pathways in the phenylpropanoid pathway, in which ferulate pathway is the major pathway leading the production of scopoletin as shown in figure 6.1 (1) (Bayoumi et al., 2008). When scopoletin is oxidised, it forms a blue complex resulting in discoloration of the roots (Miller et al., 1975). Reduction of scopoletin levels in transgenic cassava, achieved through inhibiting the gene encoding feruloyl CoA 6′-hydroxylase responsible for the biosynthesis of scopoletin (Figure 6.1.A) via RNAi silencing, significantly delayed PPD (Liu et al., 2017), confirming the vital role of scopoletin in the PPD process.

Under normal condition, plants convert scopoletin to its glucoside form, scopolin via glucosylation and release scopoletin from scopolin in response to stress conditions such as wounding or microbial attack. From this pathway, it looks obvious that the interconversion of scopoletin and scopolin influences the scopoletin status in the plant cells under stress conditions including wound stress that triggers PPD in cassava. However, the role of this interconversion in cassava PPD response is still unclear. Therefore, modifying this pathway in cassava and investigating its effects on scopoletin and scopolin accumulation as well as on PPD response will facilitate a better

understanding of cassava PPD mechanisms and may suggest a solution to tackle cassava PPD problem.

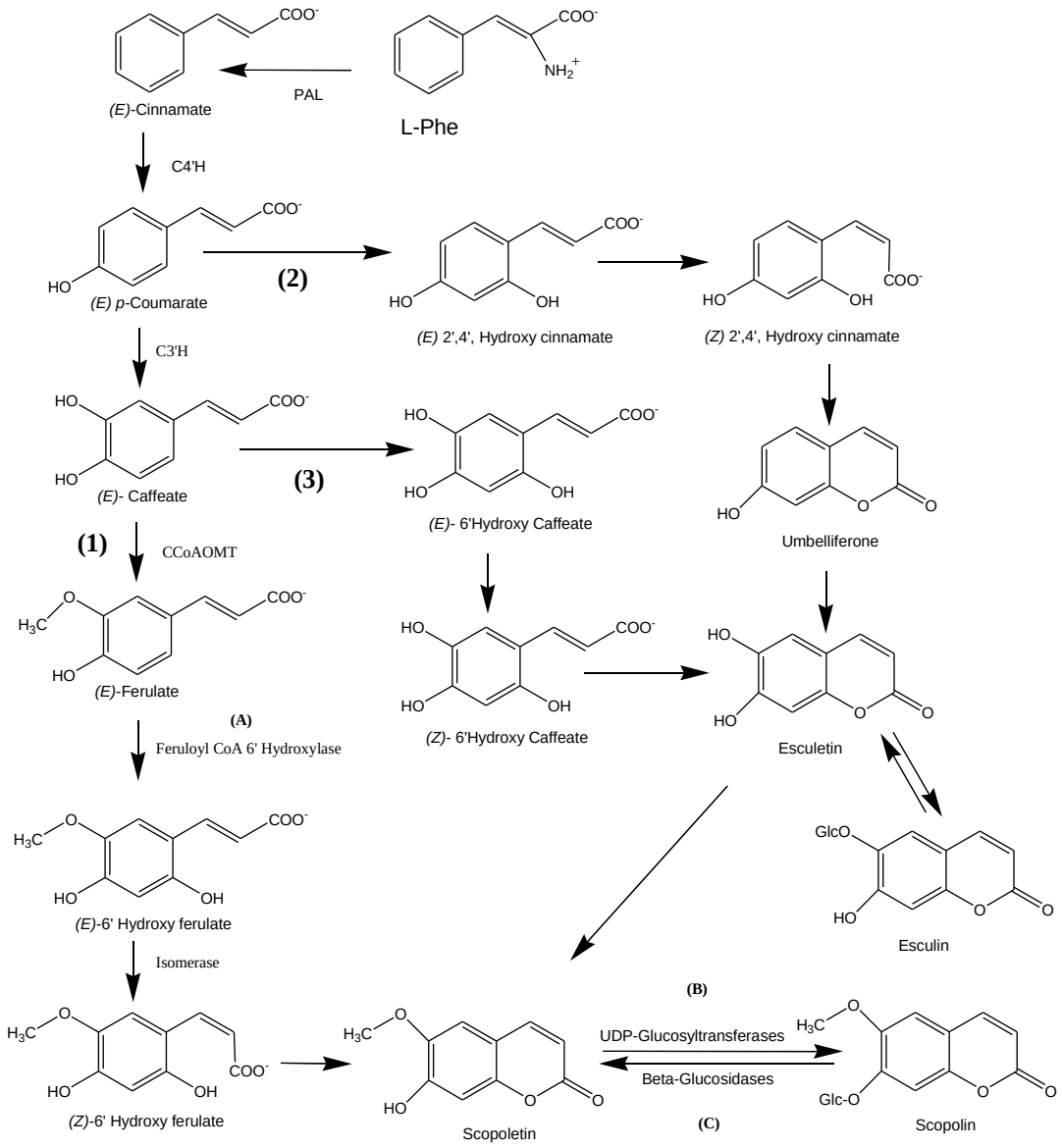


Figure 6.1. General phenylpropanoid metabolism leading to the production of scopoletin and scopolin in plants. The figure was re-drawn and slightly modified from (Bayoumi et al., 2008).

The interconversion of scopoletin and scopolin involves two reactions:

glucosylation of scopoletin regulated by scopoletin-glucosyltransferase (Scopoletin-GT) (Figure 6.1.B) and de-glucosylation of scopolin regulated by scopolin beta-glucosidase (Scopolin-BG) (Figure 6.1.C). While, this chapter focuses on the glucosylation step, the de-glucosylation of scopolin by scopolin-beta-glucosidase will be discussed in the next chapter.

Glucosylation is often the last step in the biosynthesis of natural compounds and plays an important role in plant stress management (Lanot et al., 2006, Bowles et al., 2005, Vogt and Jones, 2000). It can alter phenylpropanoid solubility, stability, toxic potential also influence compartmentalisation and biological activity (Le Roy et al., 2016). In scopoletin biosynthesis, it converts scopoletin to its glucoside, scopolin. Down-regulation of the gene encoding scopoletin-GT in tobacco-*togt*1 led to decreased not only scopolin but also, surprisingly, scopoletin (Chong et al., 2002). In addition, our current study in a knock-out mutant of a homologous gene to *togt*1 also showed that scopoletin and scopolin levels were significantly decreased in Arabidopsis mutant (Chapter 4). These results suggested the possibility that similar manipulation of the homologous cassava gene(s) could also alter both scopoletin and scopolin contents in transgenic cassava roots. Therefore, in this chapter, we attempted to emulate this strategy in cassava and investigate to what extent down-regulation of cassava scopoletin-GT would affect scopoletin and scopolin accumulation in cassava roots and PPD response. Our hypothesis was that inhibiting the conversion of scopoletin to scopolin could lead to a) a reduced scopolin level and increased scopoletin accumulation as a result of inhibiting the glucosylation step, or b) a reduced both scopoletin and scopolin content as was shown in tobacco and Arabidopsis. Either options would affect PPD response in cassava.

6.2. Aim of study and research strategies

The aim of this chapter was to investigate the effects of down-regulation of

cassava scopoletin-GT, *MeSGT*1, via an RNAi construct on scopoletin and scopolin content and PPD response in cassava.

The following strategies were used to achieve the aim of the study:

1. Assess the effect of the transgene on general plant morphology and phenotype.
2. Assess PPD of the roots by MATLab-based imaging software and select two best lines for further analysis.
3. Analyse coumarins content including scopoletin, scopolin, esculetin and esculin in wild type and selected transgenic cassava by LC-MS.
4. Analyse relative expression of *MeSGT*1 in wild type and selected transgenic cassava by qRT-PCR.

6.3. Results

6.3.1. Phenotypic traits of glasshouse-grown *MeSGT*1 RNAi-transgenic cassava

Plant height and plant growth rate. Eight independent *MeSGT*1 RNAi-expressing transgenic lines driven by either constitutive Cauliflower Mosaic Virus 35S (CaMV-35S) or root-specific *StPATATIN* promoters were grown in a 1-L pot under glasshouse conditions (Figure 6.2). In order to monitor plant growth, plant height was measured at three time points at 2, 4 and 6 months before harvesting the plants for root analysis (Figure 6.3). The statistical analysis was performed on the data basis of transgenic lines and wild type::GUS. We used two types of cassava wild type as controls: Wild type1, non-transformed plant, which was sub-cultured from *in vitro* collection and wild type::GUS, transformed plant carrying GUS Plus gene, which was generated from FECs. After 6 months, six RNAi-expressing lines including 35S::*MeSGT*1 RNAi-expressing line 1, 2, PAT::*MeSGT*1 RNAi-expressing line 1,2,3 and 4 showed higher growth (plant height) than the wild type with highly statistically significant different (P value < 0.01).

Figure 6.2. Cassava plant growth in the glasshouse after 6 months under controlled condition of 28-30 ºC, >50% Relative humidity and 16 h daylight.

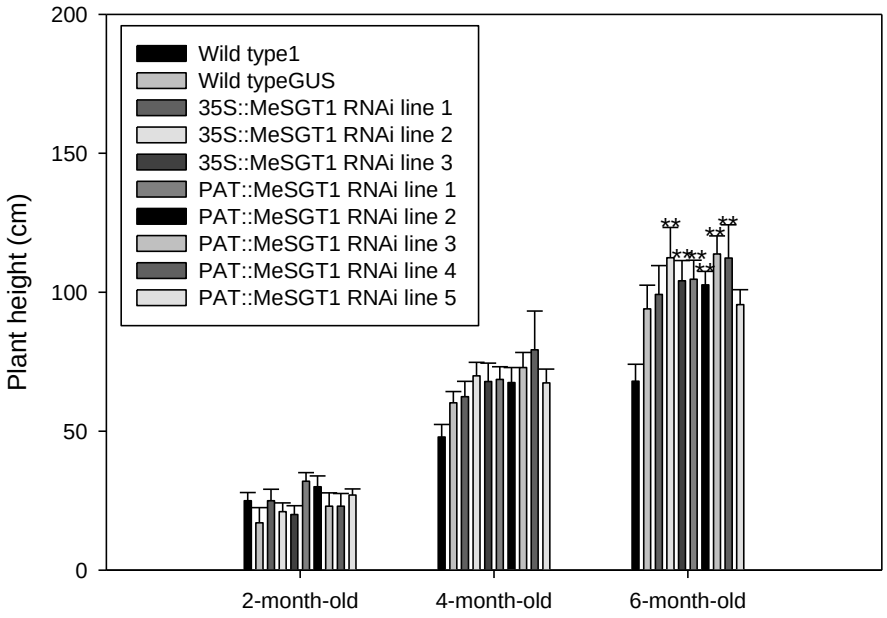


Figure 6.3. Evaluation of plant height of *MeSGT*1 RNAi-expressing transgenic cassava lines against cassava wild type. Data were collected from 10 plants per line at three time points (month 2, 4 and 6). Plant height is given as average ± SD. SD refers to the standard deviation of 10 samples or replicates. **: highly significant different (P value < 0.01) to wild type::GUS.

Plant growth rate was also evaluated by comparing data of plant height between month 2 and month 6 (Figure 6.4). There was a variation in the

growth rate (cm per month) between wild type and transgenic lines. Transgenic lines showed higher growth rate compared to wild type1 but similar to wild type::GUS. These results were indicating that down-regulation of *MeSGT*1 in the cassava genome does not negatively affect the plant height/growth.

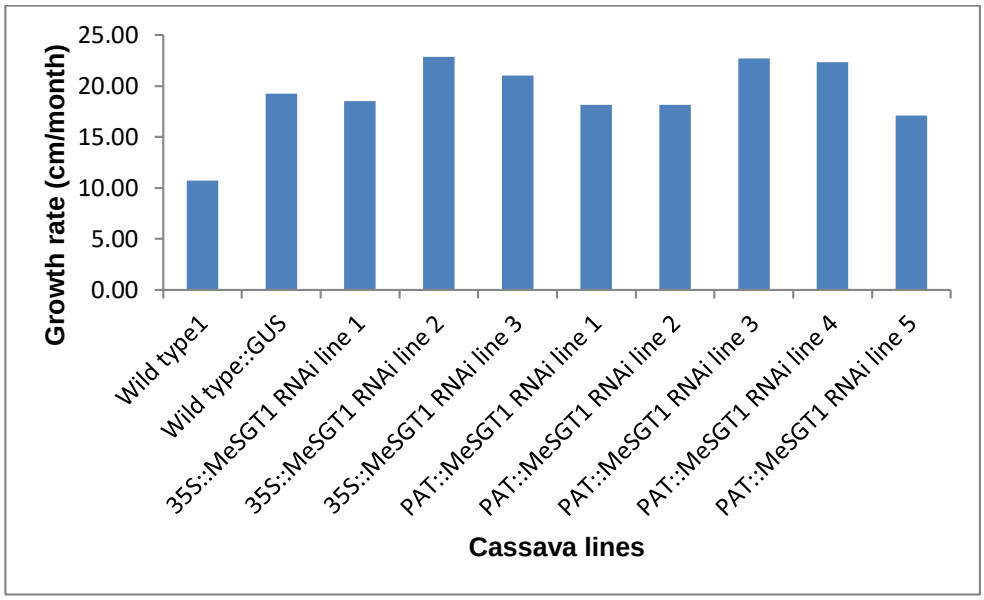


Figure 6.4. Growth rate of *MeSGT*1 RNAi-expressing transgenic cassava lines against cassava wild type cv. 60444. The growth rate (cm per month) was calculated based on plant height data at month 2 vs month 6.

Stem diameter and leaf shoot. Small pot (1-L)-grown plants did not show significant phenotypic variation. However, the *MeSGT*1-RNAi transgenic lines showed morphological variation in stem diameter (Figure 6.5) and leaf shoot appearance (Figure 6.6) when they were grown in a bigger pot (10 L) where the plants could grow normal as in the field. In the bigger pot, we only grew wild type::GUS as a control due to space limit in the glasshouse.

1 2 3 4 5 6 7 8 9

Figure 6.5. Variation of stem diameter between *MeSGT*1 RNAi-expressing transgenic lines and wild type. 1. Wild type::GUS, 2. 35S::RNAi line 1, 3. 35S::RNAi line 2, 4. 35S::RNAi line 3, 5. PAT::RNAi line 1, 6. PAT::RNAi line 2, 7. PAT::RNAi line 3, 8. PAT::RNAi line 4 and 9. PAT::RNAi line 5. The plants were grown in a bigger pot where they grew normal as in the field and were harvested after 12 months. The diameter of stem ranged from 5 mm to 18 mm.

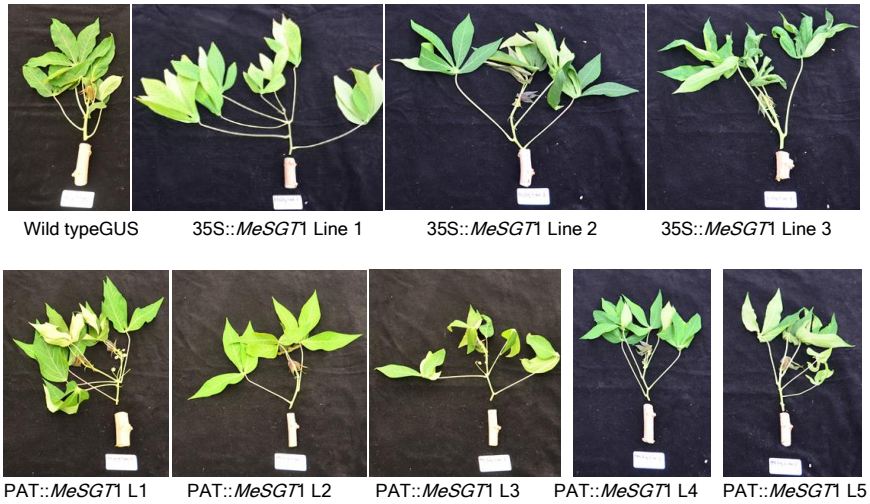


Figure 6.6. Variation of shoot appearance between *MeSGT*1 RNAi-expressing transgenic lines and wild type. The plants were grown in a 10-L pot where they grew normal as in the field and were harvested after 12 months.

Potential yield: root number and root weight. For these analyses, 6-month-old cassava were evaluated for their potential yield including root number and root weight prior to PPD assessment (Figure 6.7). There was no significant different in root number between transgenic lines and both wild type: wild type1 and wild type::GUS, the average root number was about 3 roots per plant. Although the average of root number was similar, some of transgenic lines showed statistically significantly higher in the root

weight compared to the wild type (Figure 6.8), which may be useful for high yield line selection.

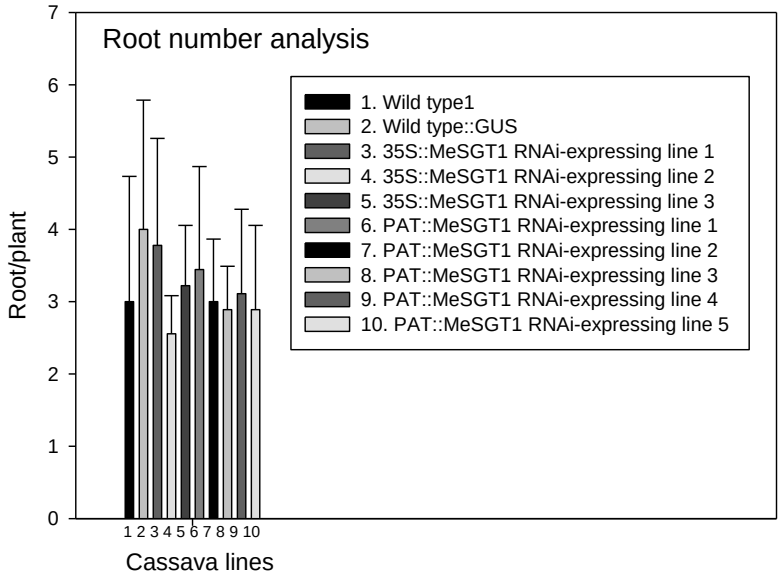


Figure 6.7. Analysis of root number of *MeSGT*1 RNAi-expressing transgenic lines. Root number is given as average ± SD. SD refers to the standard deviation of 27 plants.

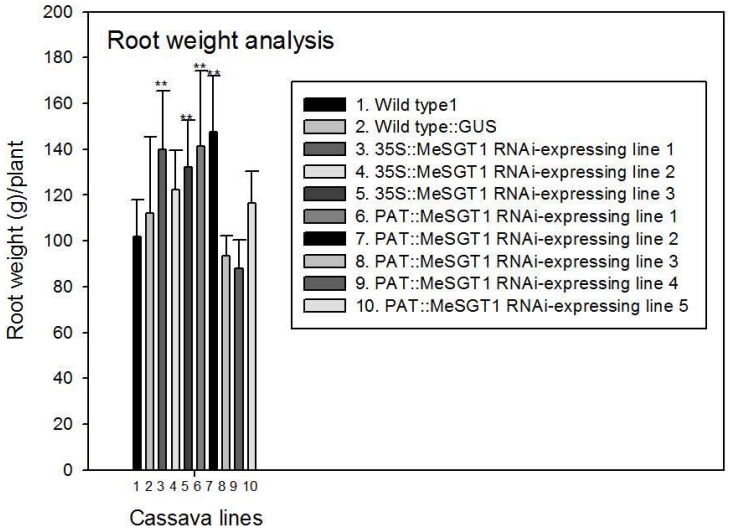


Figure 6.8. Analysis of root weight of *MeSGT*1 RNAi-expressing transgenic lines. Root weight is given as average ± SD. SD refers to the standard deviation of 27 plants.

Dry matter content (DMC). Most of the transgenic lines showed higher dry matter content (DMC) ranging from 33% to 42% than wild type of 29% (Figure 6.9). The transgenic lines exhibited negative correlation between DMC and the degree of PPD (% discoloration). A high DMC is one of the important traits in cassava, being preferred by farmers. However, this trait has a positive correlation with PPD, which has inhibited breeding for low PPD (Chavez et al., 2005).

Since we grew plants in small pots (1-L), the roots could not develop fully as they do in the field (Figure 6.10). The root shape also made the harvest and selection of appropriate roots for PPD assessment difficult, as some roots had to be broken to detach them from the stem. However, as they grew well, we managed to obtain sufficient roots sample for PPD assessment.

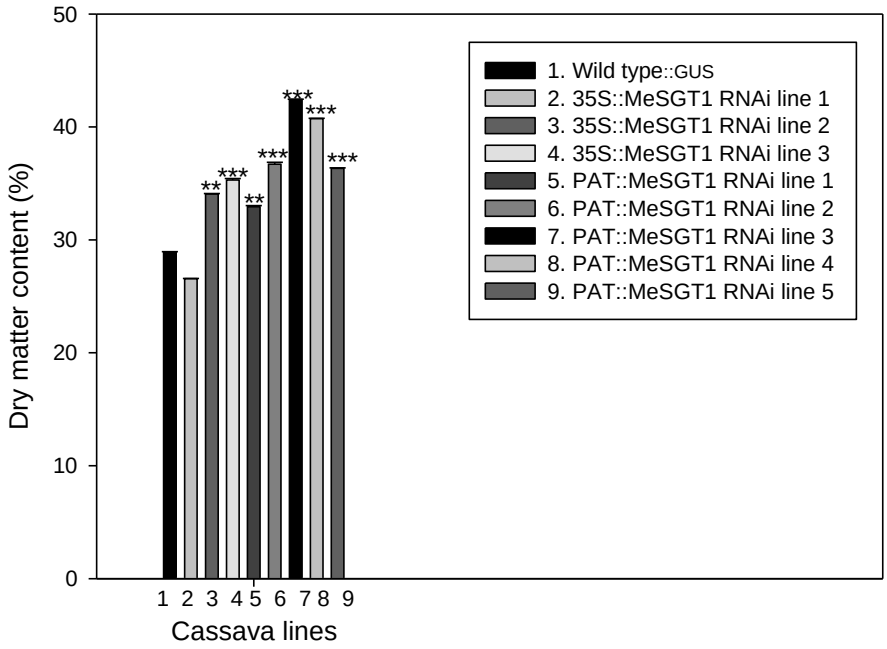


Figure 6.9. Dry matter content analysis of *MeSGT*1-RNAi transgenic lines against wild type::GUS. Dry matter content (DMC) is given as % mean of three biological replicates. **: statistically significant at P value < 0.01. ***: highly statistically significant at P value < 0.001.

Figure 6.10. Variation in root shape of wild type and RNAi expressing-transgenic cassava after 6 months of planting. Three plants were harvested and treated as one biological replicate to obtain enough root material for PPD assessment.

6.3.2. Effects of down regulation of *MeSGT*1 on cassava PPD

6.3.2.1. Down regulation of MeSGT1 reduces PPD

In this assay, we selected roots that had similar sizes for PPD assessment in order to minimise standard error due to root size variation. The results shown in figure 6.11 revealed that PPD response given as % root discoloration of eight *MeSGT*1 RNAi-expressing transgenic lines were highly significantly lower than that of control wild type (P value < 0.001). The *MeSGT*1 down regulated-transgenic lines showed a dramatic decrease in % discoloration ranged from 4.96 ± 2.23 % to 16.32 ± 8.48 % compared to wild type::GUS which was 41.80 ± 6.47 % or about 60 to 88 % reduction. Although the two wild type lines; wild type1 and wild type::GUS, showed significant different in plant growth, PPD assay revealed they had similar response to PPD. The use of different promoters showed no significant

different in the PPD assessment results of the *MeSGT*1-RNAi lines.

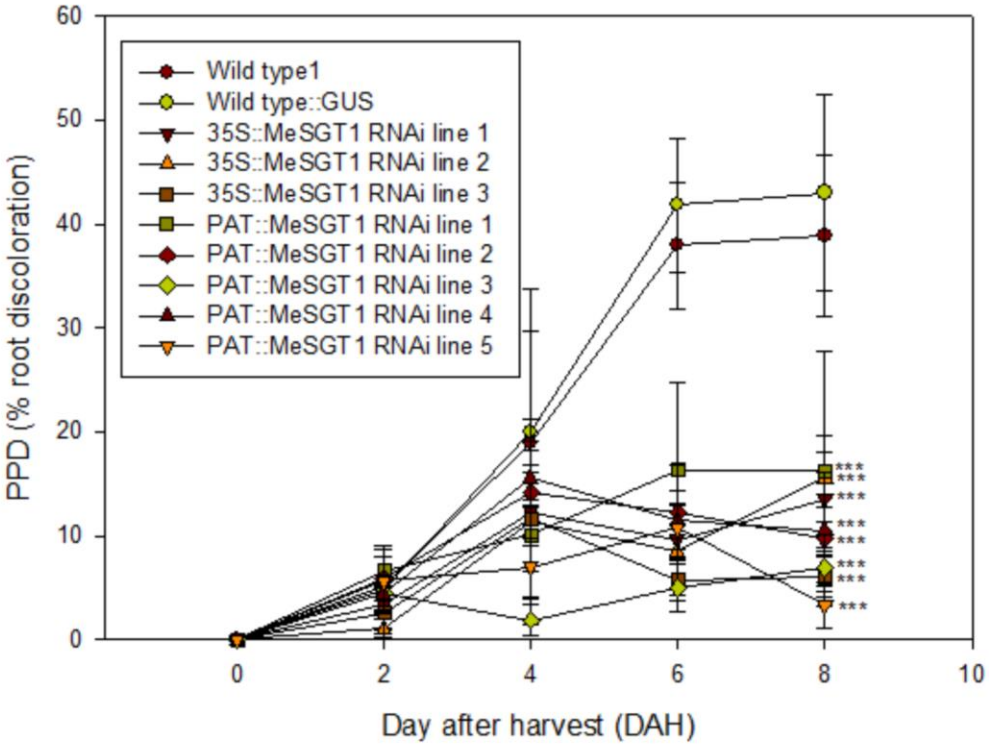


Figure 6.11. PPD assessment of *MeSGT*1 RNAi-expressing transgenic lines against wild type at 2, 4, 6 and 8 days after harvest. All *MeSGT*1 RNAi-expressing transgenic lines showed a significant lower in % discoloration than wild type. PPD is given as average of % discoloration (PPD score x 100) ± SD. % discoloration of root is normalised to % discoloration at time 0. SD refers to the standard deviation of three biological replicates. ***: highly significant (P value < 0.001).

PPD rate (day^{-1}) and extrapolation of delayed PPD time

PPD rate (day^{-1}) was calculated according to Moyib et al. (2015). The results showed that PPD rate (day^{-1}) varied between the *MeSGT*1 RNAi-expressing transgenic lines ranging from 5 % to 14 % day^{-1}. An estimated delayed PPD time in transgenic lines was predicted between 2 and 7 days (Table 6.1) using formula in section 2.12.9.3. However, this is only an extrapolation of past % discoloration trends and assumes that the PPD rate remained constant. To test this prediction, more research such as extended PPD induction up to 2-3 weeks is required.

Table 6.1. Analysis of the PPD rate (day^{-1}) and estimated delayed PPD time (day) in *MeSGT*1 RNAi-expressing transgenic lines.

No	Line	PPD rate (day^{-1})	Estimated delayed PPD (day)
1	Wild type::GUS	34.21	0.00
2	35S::MeSGT1 RNAi line 1	11.08	2.91
3	35S::MeSGT1 RNAi line 2	11.11	3.00
4	35S::MeSGT1 RNAi line 3	6.73	5.36
5	PAT::MeSGT1 RNAi line 1	14.04	1.82
6	PAT::MeSGT1 RNAi line 2	10.97	2.70
7	PAT::MeSGT1 RNAi line 3	5.06	7.28
8	PAT::MeSGT1 RNAi line 4	11.16	2.72
9	PAT::MeSGT1 RNAi line 5	6.52	4.76

Root images showed less % discoloration in roots of MeSGT1 RNAi-expressing transgenic lines

A significant reduction was shown in root discoloration of *MeSGT*1 RNAi-expressing transgenic lines compared to wild type (Figure 6.12). In here, we presented an example of root discoloration appearances between wild type and the selected transgenic lines with the lowest % discoloration: 35S::*MeSGT*1 RNAi line 3 and PAT::*MeSGT*1 RNAi line 3 during PPD.

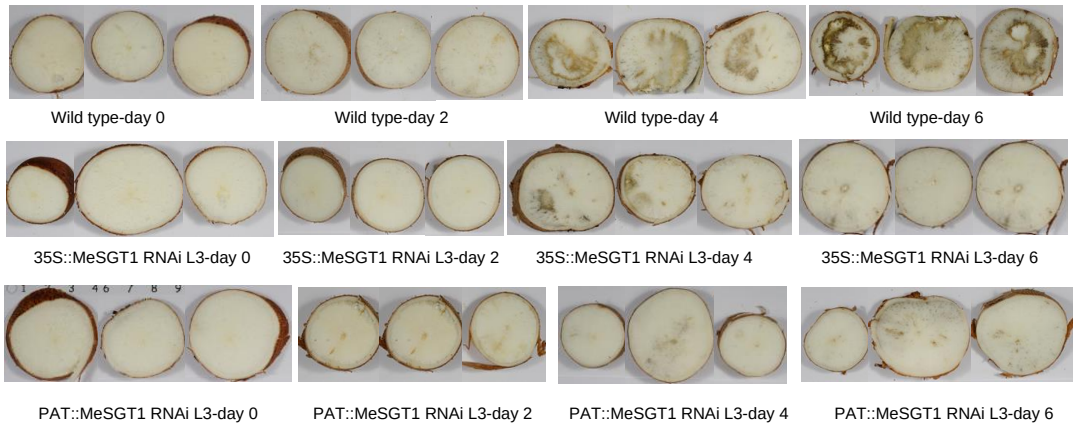


Figure 6.12. Root discoloration changes of the selected *MeSGT*1 RNAi-expressing transgenic lines over PPD time course. The diameter of sliced root was between 15 mm to 30 mm.

6.3.2.2. Down regulation of MeSGT1 reduces scopoletin and scopolin in transgenic lines

To investigate further the effects of down regulation of *MeSGT*1 on PPD-induced biochemical response, we measured scopoletin, scopolin, esculetin and esculin content in wild type and transgenic cassava lines. Of eight transgenic lines, we only selected two best lines based on the lowest % root discoloration (Table 6.1): 35S::MeSGT1 RNAi line 3 (5.75 ± 1.48) and PAT::MeSGT1 RNAi line 3 (4.96 ± 2.23) due to time- and cost-constraints. Quantification by LCMS presented in figure 6.13 showed that scopoletin and scopolin content in fresh roots (day 0) and deteriorating ones (day 6) of the selected transgenic lines were much lower than wild type. During PPD onset, wild type cassava showed a constant increase in scopoletin levels over PPD time while two transgenic lines demonstrated different responses in terms of scopoletin accumulation peak. Scopoletin accumulation in transgenic lines initially increased and reached the peak at between day 2 and 4 then decreased after day 4, while in wild type, it kept increasing until day 6. In 35S::MeSGT1 RNAi line 3, scopoletin accumulation reached the peak of 2.77 ± 0.35 ng/mg fresh weight (FW) root at day 4, and 6.49 ± 1.16 ng/mg FW root in PAT::MeSGT1 RNAi line 3 at day 2. Except for

PAT::*MeSGT*1 line 3 at day 2, scopoletin levels in all transgenic lines was significantly lower than wild type over PPD periods. In terms of scopolin, wild type and transgenic showed the same trend where scopolin accumulation increased and reached maximum levels at day 2. Surprisingly, scopolin was detected at even higher levels than wild type from day 2 to day 4. Scopolin accumulation increased dramatically at day 2 and reached the peak of 14.03 ± 2.92 ng/mg FW root in 35S::MeSGT1 RNAi line 3, and 11.83 ng/mg FW root in PAT::MeSGT1 RNAi line 3.

We also detected other coumarins: esculetin and its glucoside, esculin in the roots during PPD. Overall, the levels of esculetin and esculin in *MeSGT*1 RNAi transgenic lines were significantly lower than those in wild type (Figure 6.14) and the two compounds showed different patterns over PPD time course. In wild type and transgenic lines, esculetin accumulation increased and reached the peak at between day 2 and day 4. Esculin, however, continued increasing until day 6 in wild type whilst in *MeSGT*1-RNAi transgenic lines, there was an increase but not as much as wild type.

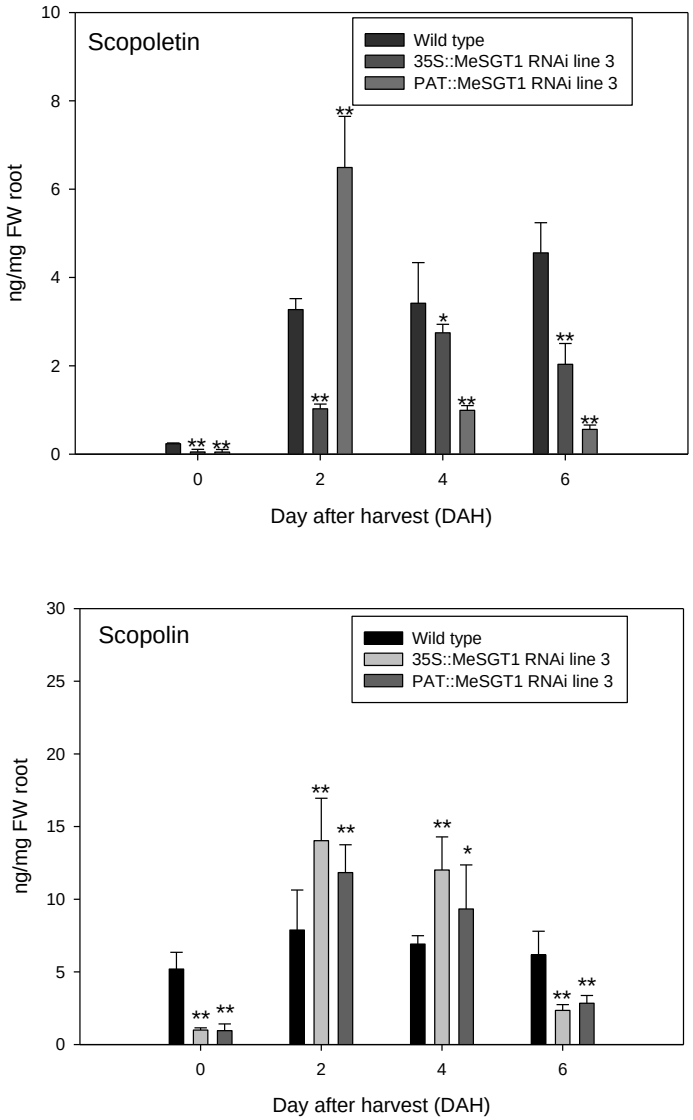


Figure 6.13. Changes of scopoletin and scopolin accumulation during PPD progress in *MeSGT*1 RNAi-expressing transgenic lines against wild type. The transgenic lines showed a significant reduction in scopoletin and scopolin accumulation at day 6 compared to wild type. The value is given as average of scopoletin or scopolin content ± SD (ng/mg FW root). SD refers to the standard deviation of three biological replicates. **: highly significant (P value < 0.01).

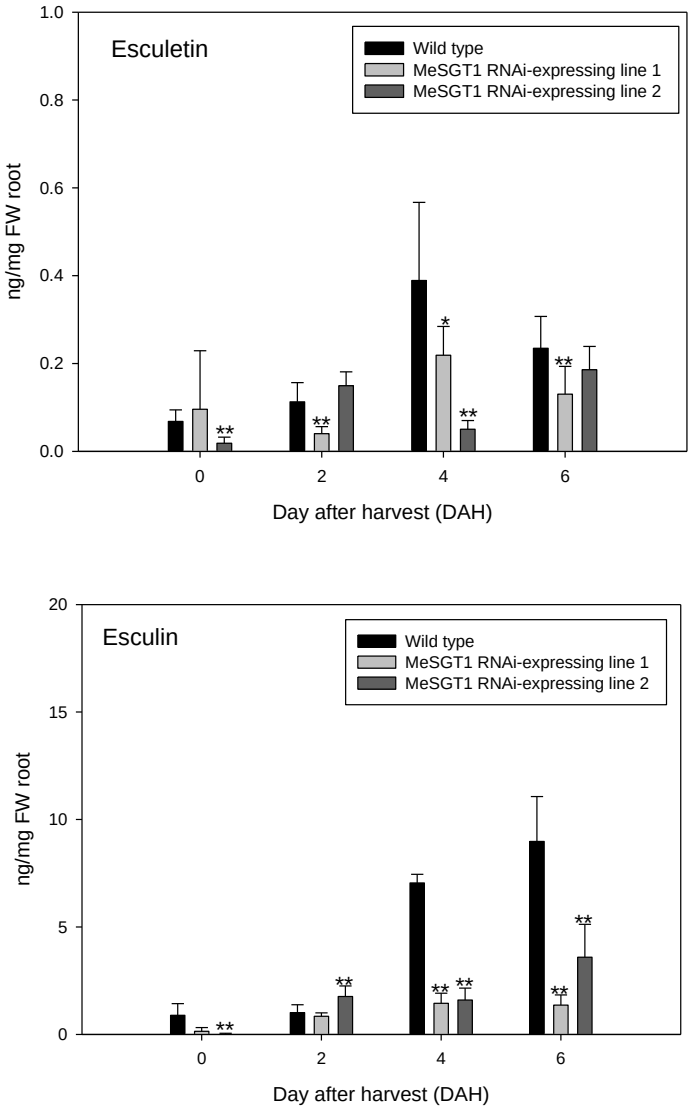


Figure 6.14. Changes of esculetin and esculin accumulation during PPD progress in *MeSGT*1 RNAi-expressing transgenic lines against wild type. The transgenic lines showed a significant reduction in scopoletin and scopolin accumulation at day 6 compared to wild type. The value is given as average of scopoletin or scopolin content ± SD (ng/mg FW root). SD refers to the standard deviation of three biological replicates. **: highly significant (P value < 0.01).

6.3.3. Scopoletin showed positive correlation to PPD

To investigate the correlation between scopoletin/scopolin and PPD, we plotted data of % discoloration against scopoletin or scopolin content over

PPD time course. As shown in figure 6.15, scopoletin showed positive correlation with PPD progress (% discoloration), while scopolin did not (Figure 6.16), indicating that scopoletin might play a direct and important role in PPD response in terms of % root discoloration.

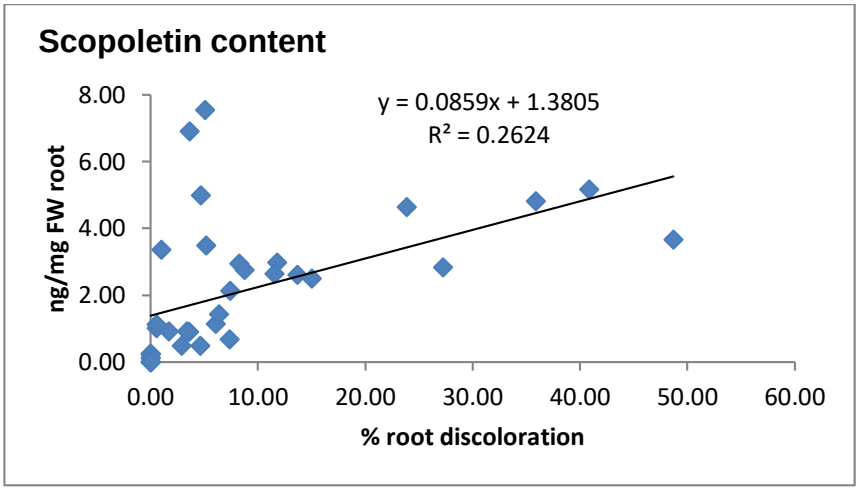


Figure 6.15. Correlation analysis between scopoletin content and PPD. Scopoletin is strongly correlated to PPD (R^2 = 0.2624). Data presented are average of three biological replicates data over time course of PPD (day 0, 2, 4 and 6).

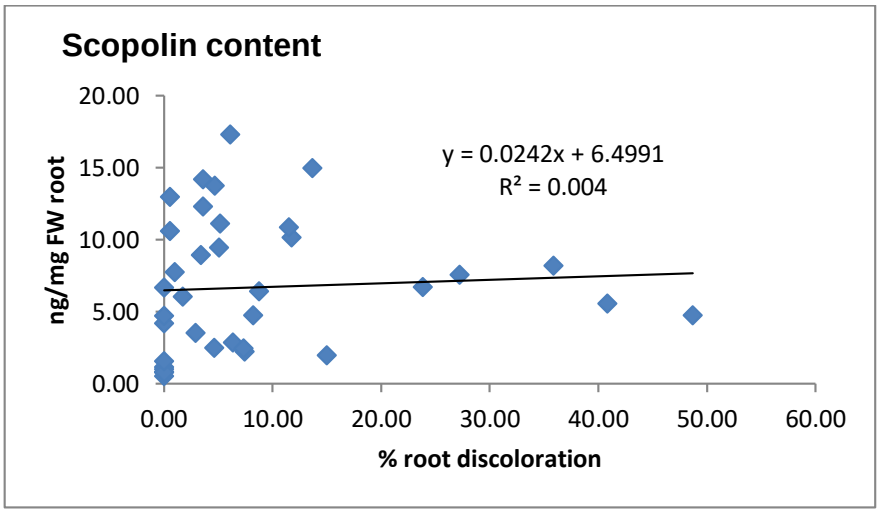


Figure 6.16. Correlation analysis between scopolin content and PPD. Scopolin shows weak correlation to PPD (R^2 = 0.004). Data presented are average of three biological replicates data over time course of PPD (day 0, 2, 4 and 6).

6.3.4. Changes of *MeSGT*1 gene expression levels during PPD

6.3.4.1. Verification of primer efficiency for qRT-PCR

The expression of *MeSGT*1 during PPD progress was analysed by qRT-PCR using a comparative C_T experiment with cDNA from roots. Three primers pairs with estimated PCR products ranging from 50 bp to 147 bp were designed and were tested for their amplification efficiency using a relative standard curve experiment prior to comparative C_T experiment. The C_T values, PCR cycle number of which the fluorescence level meets the threshold, were plotted against quantity of cDNA to create standard curve (Figure 6.17). The average amplification efficiency varied among three primers pairs (Table .6.2). Primer 2 showed best efficiency of 99.989%, therefore it was then used for comparative C_T experiment.

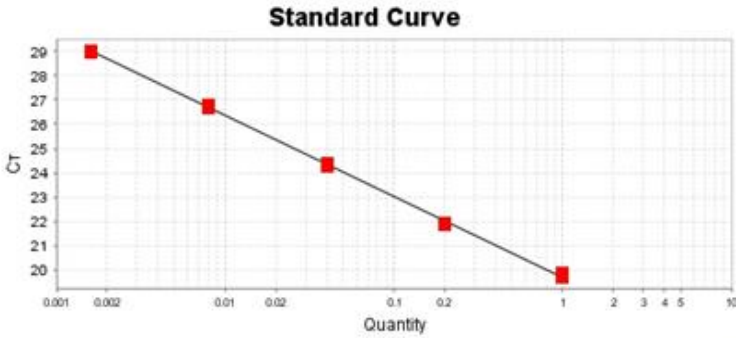


Figure 6.17. Standard curve of *MeSGT*1 obtained from primer 2 with best efficiency value of 99.989%.

Table 6.2. Summary of relative standard curve experiment.

No	Primer	Slope	R^2	Efficiency %
1	Reference gene:Ubq10	-3.409	0.996	96.498
2	*MeSGT*1 Primer 1	-3.528	0.857	119.731
3	*MeSGT*1 Primer 2	-3.322	0.999	99.989
4	*MeSGT*1 Primer 3	-3.021	0.852	114.313

6.3.4.2. Expression profiling (C_T values) of *MeSGT*1 gene during PPD

The expression level of *MeSGT*1 in wild type and transgenic lines were presented in C_T values. Figure 6.18 shows that the target mRNA was detected less in both RNAi-expressing transgenic lines compared to control wild type in the fresh roots and during PPD progress. The pattern of C_T values during PPD time course showed that the *MeSGT*1 was highly significant up regulated at day 4.

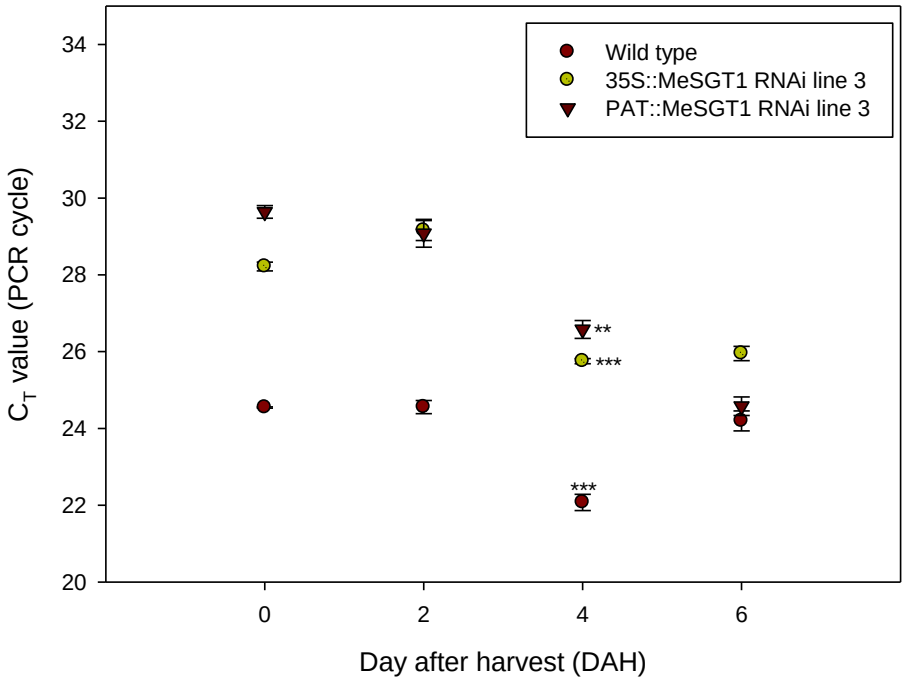


Figure 6.18. Expression level of *MeSGT*1 gene in root samples of RNAi transgenic lines and wild type during PPD progress. MeSGT1 gene is up regulated in all samples. Data given is an average of C_T values ± SD. SD refers to standard deviation of three biological replicates and each run with three technical replicates. **: highly significant at P value < 0.01 and ***: highly significant at P value < 0.001.

6.3.4.3. Relative expression of *MeSGT*1 during PPD

The relative expression of *MeSGT*1 was calculated with the Livak method or $\Delta\Delta C_T$ method and was presented as relative quantities-RQ ($2^{-\Delta\Delta CT}$) as shown in Figure 6.19. The qRT-PCR results revealed that *MeSGT*1 expression level in roots of both RNAi-expressing transgenic lines was

decreased compared to control wild type with highly statistically significant different (P value < 0.001). At day 0, the *MeSGT*1 in both transgenic lines was about 10 times less expressed compared to wild type. In 35S::*MeSGT*1 RNAi line, the expression of *MeSGT*1 was about 43 to 45 times lower than wild type at day 4 and 6, respectively. PAT::*MeSGT*1 RNAi line showed more reduction in the gene expression of *MeSGT*1 up to 91 times compared to wild type. Figure 6.20 reveals that the relative expression (RQ) of the *MeSGT*1 was positively correlated to the PPD response (% discoloration).

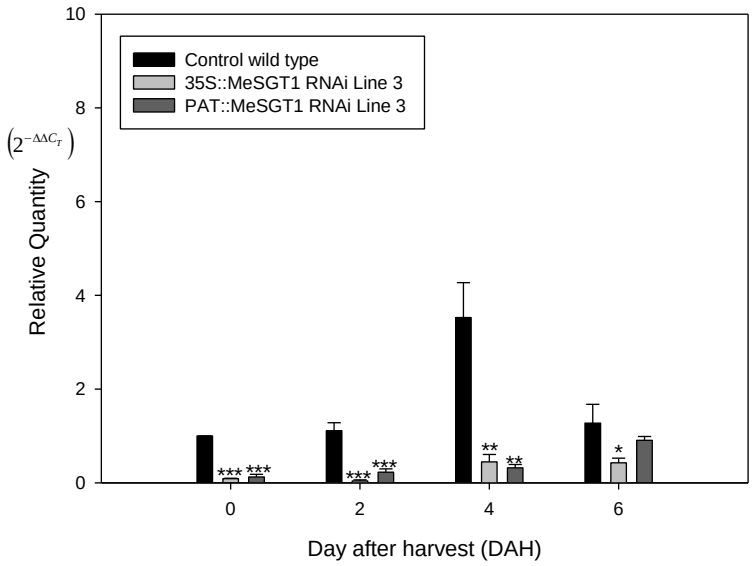


Figure 6.19. Relative quantification ($2^{-\Delta\Delta CT}$) of *MeSGT*1 in root samples of RNAi transgenic lines and wild type during PPD progress. RQ is calculated based on C_T mean values obtained from three biological replicates, normalised to the reference gene (Ubq10) and the calibrator (WT).

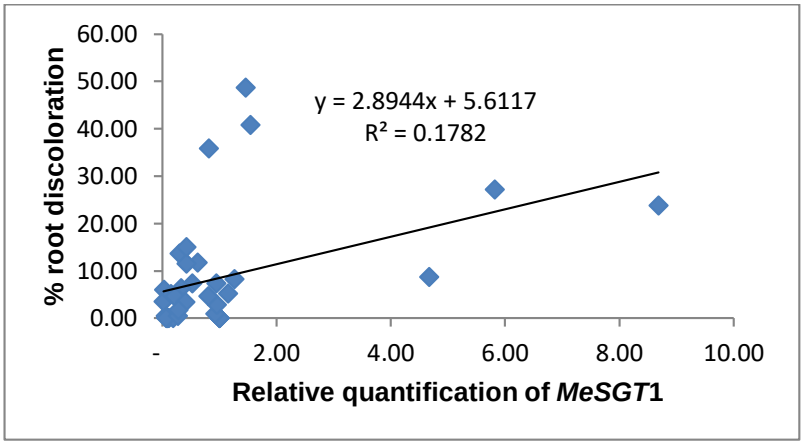


Figure 6.20. Correlation analysis of relative quantity of *MeSGT*1 and PPD response (% discoloration).

6.4. Discussion

The aim of this chapter was to investigate the effects of RNAi-mediated down regulation of the selected cassava gene encoding scopoletin glucosyltransferase (scopoletin-GT), *MeSGT*1, on scopoletin and scopolin accumulation and PPD response in transgenic cassava. This study would help to improve our understanding of PPD mechanisms and may suggest a strategy to delay PPD in cassava. The present study confirms that down regulation of cassava scopoletin-GT, *MeSGT*1, led to the decrease not only of scopolin accumulation but surprisingly also scopoletin accumulation in the fresh roots at day 0 and deteriorating roots at day 6 and significantly delayed PPD in the transgenic cassava. These findings demonstrate that the RNAi construct had successfully down regulated the *MeSGT*1 in the cassava genome and inhibited the glucosylation step.

The reduction in scopoletin level was unexpected as it was hypothesised to increase when its conversion was inhibited. However, these findings concur with other studies that show the same reduction in scopoletin and scopolin in transgenic tobacco (Chong et al., 2002) and in an Arabidopsis knock-out mutant (Chapter 4). Moreover, it provides us more evidence that knocking down the gene(s) for scopoletin-glucosyltransferase would reduce not only

scopolin but also scopoletin contents in the plant cells. The most likely explanation of the decreased scopoletin in the *MeSGT*1 RNAi transgenic lines is that suggested by Chong et al (2002). Firstly, it is well-known that phenolic compounds including scopoletin are very reactive and subject to oxidation, hence they cannot accumulate to high levels as aglycone in the plant cells. Consequently, compromising scopoletin glucosylation could enhance its degradation by cellular oxidases. Secondly, one part of the scopoletin pool may arise from the hydrolysis of its glucoside form, scopolin by beta-glucosidase. Thirdly, there is a feedback inhibition of upstream enzymes of the biosynthesis of scopoletin when scopoletin-GT is inhibited.

PPD is considered as a complex process involving many reactions started with the oxidative burst and followed by altered gene expression as well as the production of various secondary metabolites (Buschmann et al., 2000, Huang et al., 2001, Reilly et al., 2003, Reilly et al., 2007, Iyer et al., 2010). We found that four coumarins including scopoletin, scopolin, esculetin and esculin were detected at trace amounts or low levels in the fresh root of both wild type and transgenic lines. Only a few works have been done on the four coumarins analyses in cassava (Bayoumi et al., 2010, Buschmann et al., 2000, Tanaka et al., 1983). Our results agree with those literatures and indicate that in case of scopoletin and esculetin, they cannot accumulate to high levels in the plant cells due to their high reactivity and potential toxicity. Surprisingly, we found relatively lower levels of scopolin in the fresh roots of wild type cassava compared to other plant species such as Arabidopsis (Kai et al., 2008, Kai et al., 2006) and tobacco (Chong et al., 2002) and there was a consistent slight increase in its accumulation over PPD time. It suggests that a) scopolin is also biosynthesised together with scopoletin in response to wounding and b) the de-glucosylation of scopolin to scopoletin does not seem to have a significant contribution to scopoletin accumulation over PPD period. To gain more understanding of this finding, the role of de-glucosylation in cassava PPD will be discussed in chapter 7.

Scopoletin and scopolin accumulation in roots of transgenic lines increased during PPD onset and reached the peak at between day 2 and 4 after PPD induction. The peak differences in scopoletin and scopolin accumulation of the two transgenic lines suggests that PPD response, in terms of scopoletin and scopolin accumulation, is genotype dependent. Sanchez et al. (2013) also observed sort of peak variation in HMC-1 line and AM 206-5 line, where scopoletin accumulation reached the maximum levels at day 3 and day 4, respectively. The decreased scopoletin and scopolin accumulation in *MeSGT*1-RNAi transgenic cassava root positively correlates with the PPD response, demonstrating the importance of scopoletin in cassava PPD response. The involvement of scopoletin in PPD progress has been suggested in several studies (Wheatley and Schwabe, 1985, Buschmann et al., 2000, Tanaka et al., 1983, Uarrota et al., 2015, Sánchez et al., 2013, Bayoumi et al., 2010). Recent study by Liu et al (2017) has also shown that reduced scopoletin level in feruloyl CoA 6′-hydroxylase-down regulated transgenic cassava could significantly delay PPD.

In agreement with (Buschmann et al., 2000, Tanaka et al., 1983, Bayoumi et al., 2010), we also identified other coumarins: esculetin and its glucoside, esculin at very low levels in the fresh roots of both wild type and transgenic lines. During PPD period, esculetin accumulated and reached the peak at day 4, while esculin constantly increased until day 6 to similar level as scopolin. The three main components detected during PPD in this study including scopoletin, scopolin and esculin is consistent with studies by (Tanaka et al., 1983). Esculetin and esculin are produced from the other alternative pathways in the phenylpropanoid metabolism as suggested by (Bayoumi et al., 2008) who investigated three possible pathways leading to scopoletin production in cassava (Figure 6.1). Up-regulation of scopoletin-glucosytransferase gene, *MeSGT*1, during PPD period is consistent with other studies (Owiti et al., 2011, Reilly et al., 2007). This may also be indicating the importance of the glucosylation step in cassava response

towards wounding that triggers PPD.

In addition, our study shows negative relationship between dry matter content (DMC) and PPD, which is contrary with other published work (Wenham, 1995, Chavez et al., 2005). Down-regulation of *MeSGT*1 showed no significant different in overall results between transgenic lines driven by constitutive 35S promoter and root-specific PATATIN promoter. However, it is often important to use or select appropriate specific promoters in the gene silencing experiment because the effects of constitutive gene silencing throughout the plant may affect its growth and the development (Katoch and Thakur, 2013). This is the first study, to best of our knowledge, to investigate the role of the gene responsible for glucosylation of scopoletin, *MeSGT*1, in cassava PPD by modifying its expression through RNAi silencing. This chapter is one of the two strategies to study the role of interconversion of scopoletin and scopolin in cassava PPD by knocking-down cassava scopoletin-GT in glucosylation step via RNAi gene silencing. While, another strategy, which is overexpressing Arabidopsis scopolin-BG, BGLU23, in cassava will be discussed in the next chapter.

Chapter 7. Overexpression of Arabidopsis scopolin beta-glucosidase-BGLU23 reduces scopoletin and scopolin content and delays PPD in cassava

Chapter 7. Overexpression of Arabidopsis scopolin beta-glucosidase-BGLU23 reduces scopoletin and scopolin content and delays PPD in cassava

7.1. Introduction

The accumulation of hydroxycoumarins, particularly scopoletin in the cassava roots after injury or harvest has been strongly correlated with postharvest root deterioration or PPD (Liu et al., 2017, Uarrota et al., 2015, Sánchez et al., 2013, Bayoumi et al., 2010, Buschmann et al., 2000, Wheatley and Schwabe, 1985, Tanaka et al., 1983). Scopoletin forms a blue complex resulting in discoloration of the roots when it is oxidised by reactive oxygen species (ROS) generated in the early events of PPD (Miller et al., 1975, Iyer et al., 2010). Scopoletin is synthesised as part of general phenylpropanoid metabolism, while some is also released from the de-glucosylation of its glucoside, scopolin in response to stress conditions (Chong et al., 2002). In cassava, there are three possible pathways in the phenylpropanoid metabolism leading to the biosynthesis of scopoletin (Figure 7.1). Among those, the pathway via ferulate (Figure 7.1.(1)) has shown to be the major pathway that produces scopoletin in cassava, while the other two pathways that lead to the biosynthesis of esculetin before it is converted to scopoletin or esculin are minor (Bayoumi et al., 2008). In the absence of stress conditions, scopoletin is naturally converted to its glucoside, scopolin via glucosylation and scopolin will be converted back to scopoletin as a defensive compound in response to any stress conditions.

From its biosynthetic pathway, the homeostasis of scopoletin is more likely to be determined by two mechanisms: through the general phenylpropanoid metabolism and the interconversion of scopoletin and scopolin. On one hand, an RNAi-mediated gene silencing the gene encoding feruloyl CoA 6′-hydroxylase (Figure 7.1.A) responsible for a key step in the biosynthesis of scopoletin has shown to play a vital role in delaying PPD by reducing

scopoletin content in the roots (Liu et al., 2017). On the other hand, the role of the interconversion of scopoletin and scopolin that also contributes to the status of scopoletin accumulation remains undefined.

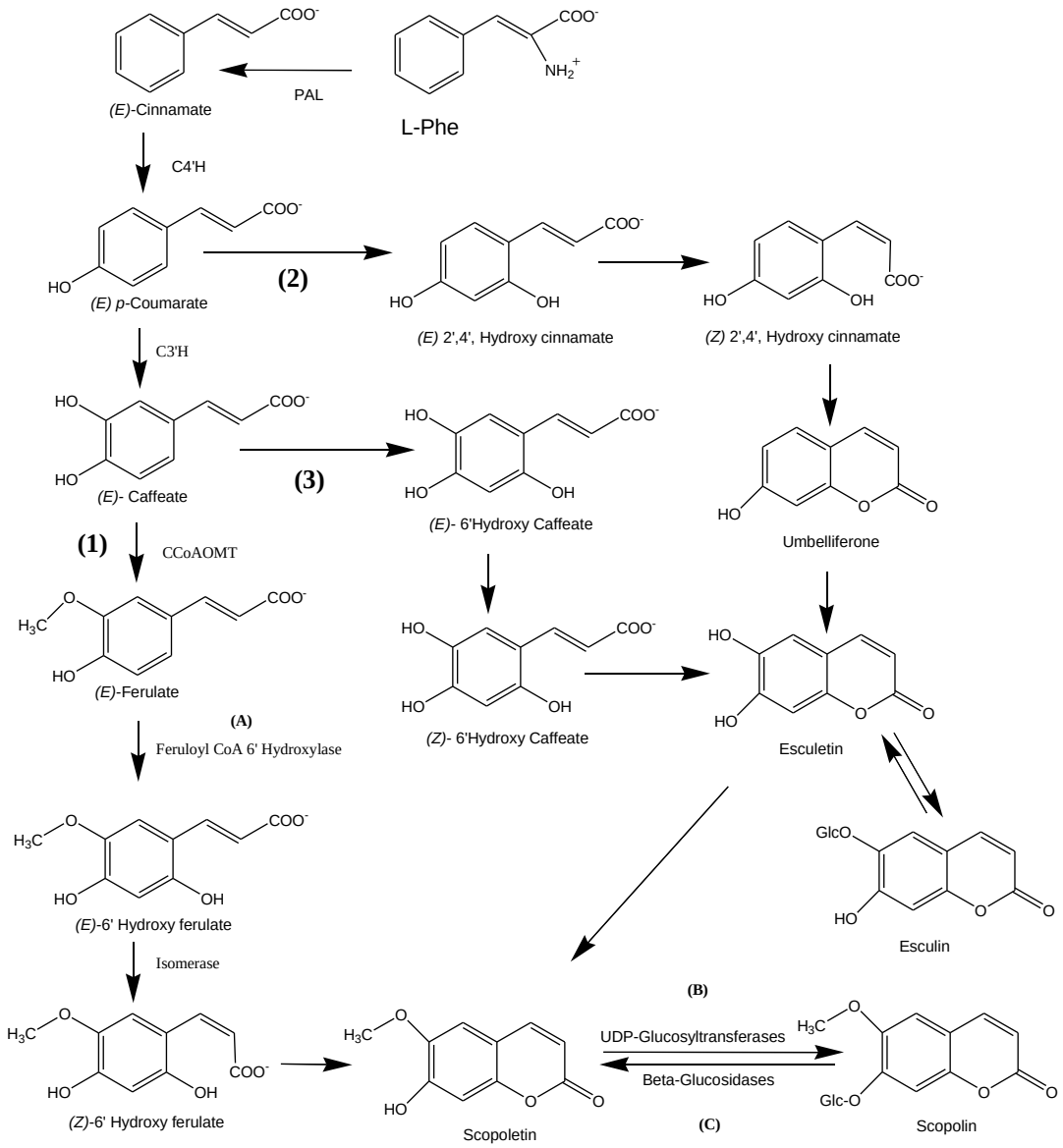


Figure 7.1. General phenylpropanoid metabolism leading to the production of scopoletin and scopolin in cassava. The figure was re-drawn and slightly modified from (Bayoumi et al., 2008).

In chapter 6, we demonstrated that disrupting the glucosylation step by knocking down the gene for scopoletin glucosyltransferase (Figure 7.1.B)

reduced scopoletin and scopolin content in cassava roots and delayed PPD significantly. This chapter, therefore, focuses on the other step, the de-glucosylation of scopolin, which is regulated by a specific scopolin-beta-glucosidase (Figure 7.1.C). This would complete our understanding of the role of the interconversion of scopoletin and scopolin in cassava PPD.

Scopolin-beta-glucosidase (scopolin-BG) is a type of scopolin-substrate specific enzyme that has ability to remove a glucose molecule from scopolin and release scopoletin. Beta glucosidases are an important class of enzymes that hydrolyse the glycosidic bond between sugars and a non-sugar moiety. In plants, they play roles in diverse functions such as lignification, catabolism of cell wall oligosaccharides, defense, phytohormone conjugate activation, and scent release, as well as in both sides of plant–microbe and plant–insect interactions (Ahn et al., 2010, Cairns and Esen, 2010). A beta glucosidase from Arabidopsis, BGLU23, was shown to possess a high and specific activity towards scopolin (Ahn et al., 2010). Since the specific cassava genes for this enzyme are not well characterised yet, the availability of a reference specific beta-glucosidase for scopolin, BGLU23, had helped us to identify homologous genes in the cassava genome. The most studied beta-glucosidase from cassava is so far only cyanogenic beta-glucosidase, linamarase (Guo et al., 2015, Kongsaeree et al., 2010).

The initial aim of this chapter was to apply the same strategy as in chapter 6 to disrupt the de-glucosylation step using an RNAi-mediated gene silencing to knock down the gene responsible for cassava scopolin-beta glucosidase (scopolin-BG). However, because it did not prove possible to identify a single cassava scopolin-BG gene within a large family of beta-glucosidases, it was decided to overexpress the specific scopolin-beta-glucosidase from Arabidopsis, BGLU23, in cassava instead. Our findings on Arabidopsis (chapter 4), cassava glucosylation modification (chapter 6),

as well as other studies in tobacco (Chong et al., 2002), showed that scopoletin content was reduced when its conversion to scopolin was inhibited, suggesting that scopoletin cannot accumulate to high level in the cells. Therefore, in this chapter we were interested in investigating how the overexpression of BGLU23 in cassava would affect scopoletin and scopolin content in transgenic cassava also PPD behaviour. Our hypothesis was that this enhanced back-conversion of scopolin to scopoletin might either a) increase scopoletin accumulation in the cells as the scopolin conversion rate is increasing and reduce scopolin accumulation, or b) even reduce both scopolin and scopoletin if the cell cannot tolerate high concentrations of scopoletin. In either case, the PPD response could be affected.

7.2. Aim of study and research strategies

This chapter aimed to investigate the effects of overexpression of Arabidopsis scopolin-BG (BGLU23) in cassava on scopoletin and scopolin content and PPD response in cassava.

The following strategies were used to achieve the aim of the study:

1. Assess the effects of the transgene on general plant morphology and phenotype.
2. Assess PPD of the cassava roots by MATLab-based imaging software and select two best lines for further analysis.
3. Analyse coumarin content including scopoletin, scopolin, esculetin and esculin in wild type and selected transgenic cassava by LC-MS.
4. Analyse relative expression of BGLU23 in wild type and selected transgenic cassava by qRT-PCR.

7.3. Results

7.3.1. Phenotypic traits of glasshouse-grown BGLU23 expressing-transgenic cassava

Plant height and plant growth rate. Six independent BGLU23-expressing transgenic cassava lines driven by either constitutive CaMV-35S or root-specific *StPATATIN* promoters were generated and were grown in a 1-L pot in the glasshouse for PPD analysis (Figure 7.2). Overall, all cassava lines including wild type and transgenic lines grew well and produced sufficient roots under glasshouse conditions.

Figure 7.2. Cassava plants growth in the glasshouse after 6 months under controlled condition of 28-30 ºC, >50% relative humidity and 16 h daylight.

To monitor plant growth, about 10 plants from each line was measured for their height periodically over 6 months of planting period at 2, 4 and 6 months (Figure 7.3). The statistical analysis was performed on the data basis of transgenic lines against wild type::GUS. After 6 months, two BGLU23-expressing transgenic lines: 35S::BGLU23-expressing line 2 and PAT::BGLU23-expressing line 3 showed lower growth than the wild type::GUS with statistically significant different (P value < 0.05). The other four transgenic lines did not show significant difference in their height compared to wild type::GUS control.

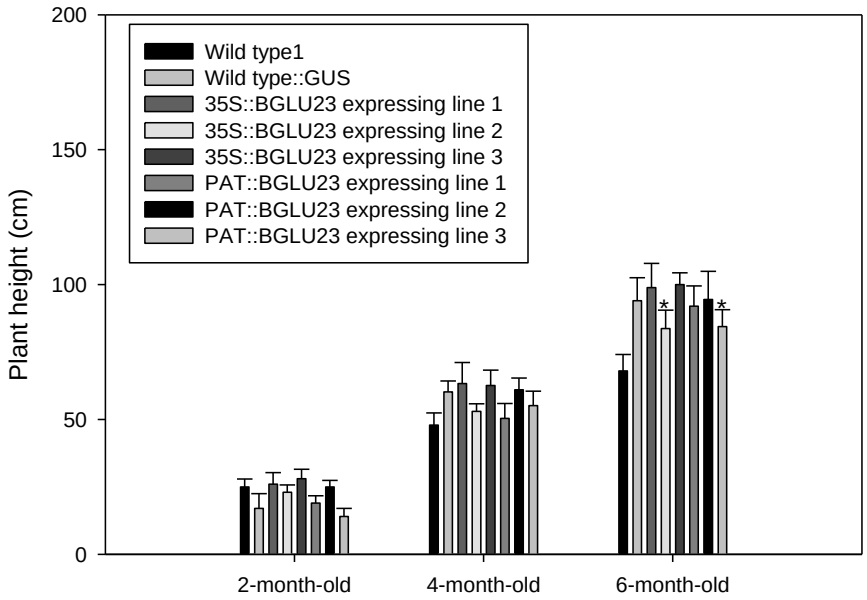


Figure 7.3. Evaluation of plant height of BGLU23-expressing transgenic cassava lines against cassava wild type cv. 60444.Data were collected from 10 plants per line at three time points (2, 4 and 6 months). Plant height is given as average ± SD. SD refers to the standard deviation of 10 replicates. We used two types of cassava wild type as control: Wild type1 (non-transformed) sub cultured from in vitro collection and wild type::GUS (transformed with GUS Plus reporter gene) generated from FECs.

The growth rate of all BGLU23-expressing transgenic lines showed no significant difference compared to wild type::GUS control (Figure 7.4). These results indicate that overexpression of BGLU23 in cassava did not negatively affect the plant height/growth. However, we observed the lowest growth rate in wild type1 control. This might be due to the nature of the plantlets as this wild type was propagated from *in vitro* collections that has gone through many subculture cycles.

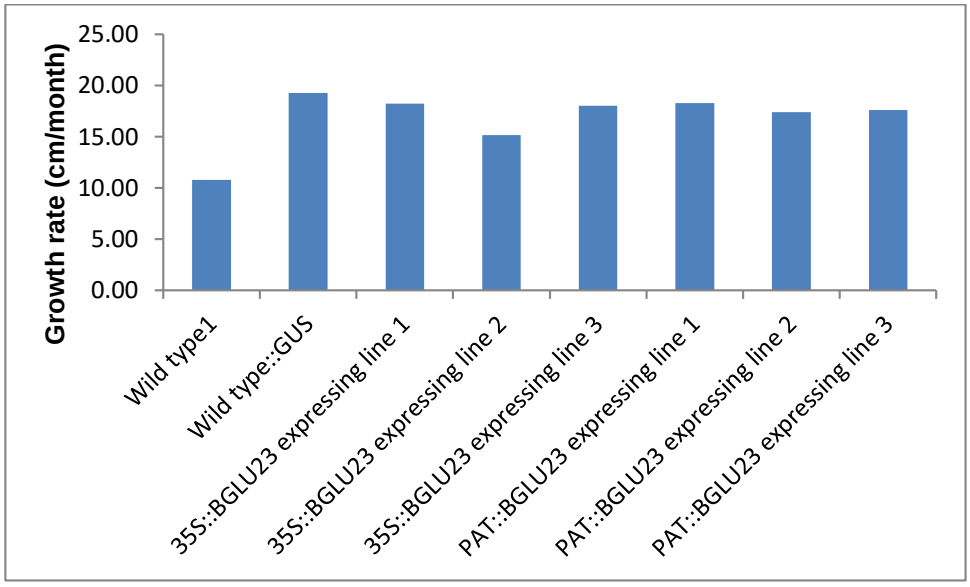


Figure 7.4. Growth rate of BGLU23-expressing transgenic cassava lines against cassava wild type cv. 60444. The growth rate (cm per month) was calculated based on plant height data at month 2 vs month 6.

Stem diameter and leaf shoot. Although all BGLU23-expressing transgenic lines showed similar plant growth/growth rate, they did show significant differences in stem diameter (Figure 7.5) and leaf shoot appearance (Figure 7.6) compared to wild type::GUS when the plants were grown in a bigger pot (10-L) where their growth resembled that found in the field. Due to space limit in the glasshouse, we only grew wild type::GUS in the bigger pot as a control.

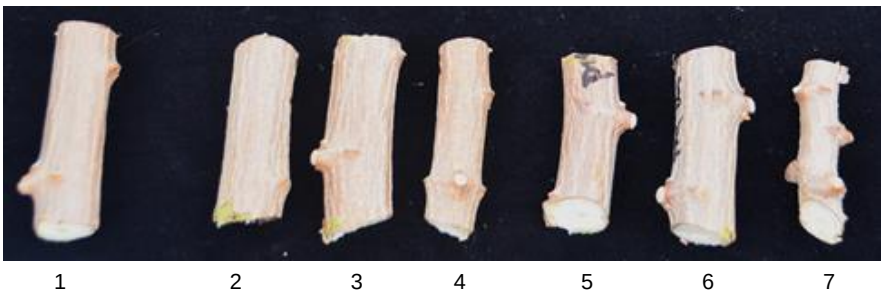


Figure 7.5. Variation of stem diameter between BGLU23-expressing transgenic lines and wild type::GUS. 1. Wild type::GUS, 2. 35S::BGLU23-expressing line 1, 3. 35S::BGLU23-expressing line 2, 4. 35S::BGLU23-expressing line 3, 5. PAT::BGLU23-expressing line 1, 6. PAT::BGLU23-expressing line 2, 7. PAT::BGLU23-expressing line 3. The plants were harvested after 12 months. The diameter of stem ranged from 8 mm to 18 mm.

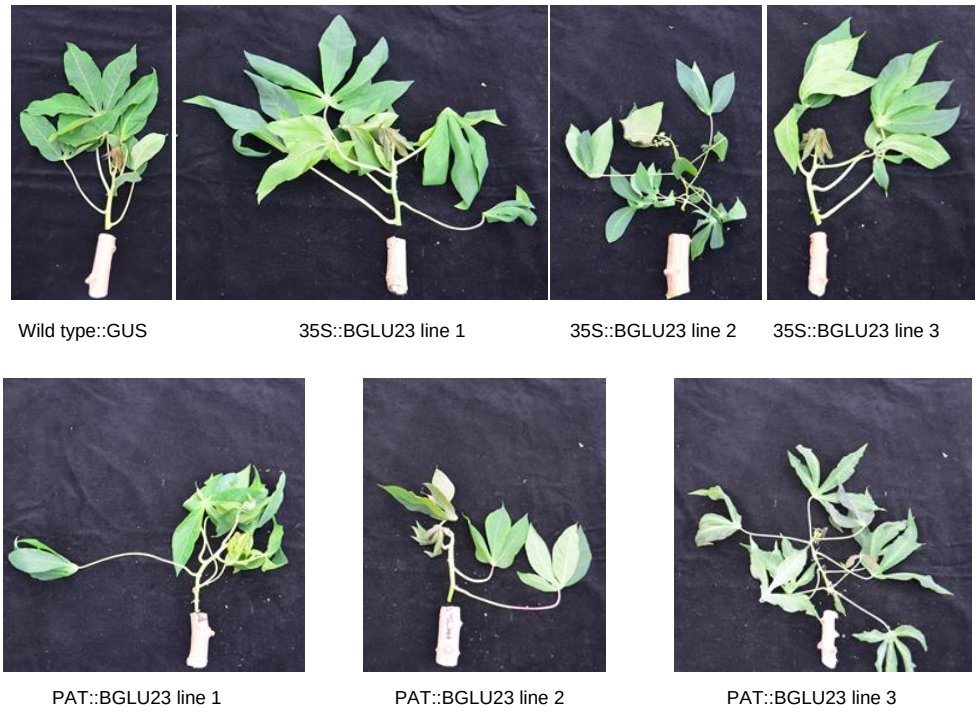


Figure 7.6. Variation of shoot appearance between BGLU23-expressing transgenic lines and wild type::GUS.

Potential yield: root number and root weight. The potential yield of the transgenic lines including root number and root weight (g per plant) were evaluated after 6 months prior to PPD assessment. The average root number of all samples was about 3-4 roots per plant (Figure 7.7). Although the average root number was similar, three transgenic lines showed significantly higher root weight than that of both wild type controls, which may be a good for high yield line selection. Moreover, we observed one transgenic line, PAT::BGLU23-expressing line 3, showing highly significantly lower in root number and potential yield than wild type (Figure 7.8). This line also showed plant morphology differences compared to wild type or other transgenic lines but interestingly showed longer delay PPD (low % discoloration), which will be discussed in the next section.

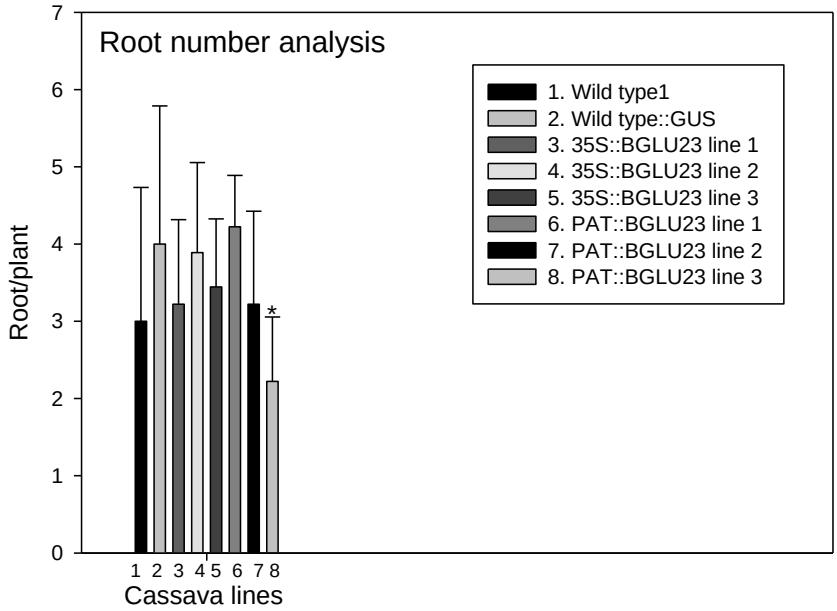


Figure 7.7. Analysis of root number of BGLU23-expressing transgenic lines. Root number is given as average ± SD. SD refers to the standard deviation of 27 plants per line.

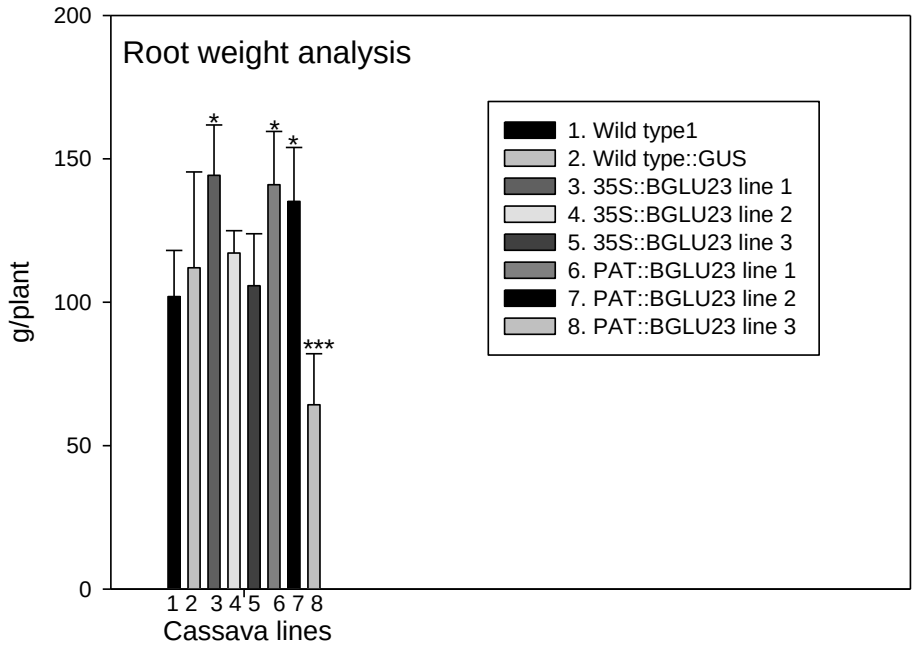


Figure 7.8. Analysis of root weight of BGLU23-expressing transgenic lines. Root weight is given as average ± SD. SD refers to the standard deviation of 27 plants per line.

Dry matter content (DMC). The majority of the BGLU23-expressing transgenic lines showed similarly low dry matter content (DMC) compared to the wild type ranged from 26% to 28% (Figure 7.9). However, two transgenic lines: 35S::BGLU23-expressing line 2 and PAT::BGLU23-expressing line showed significantly higher DMC, about 38-40% than wild type. A negative correlation between DMC and % discoloration was found in PAT::BGLU23-expressing line 3. A high DMC is one of the important agronomic traits of cassava, being preferred by farmers and industries. However, this trait has been reported to have a positive correlation with PPD, which has inhibited breeding for low PPD (Chavez et al., 2005).

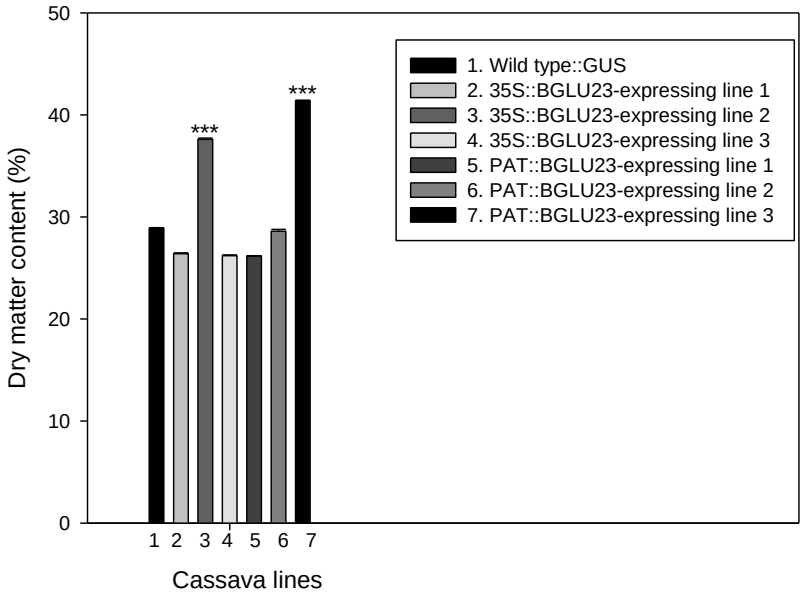


Figure 7.9. Dry matter content analysis of BGLU23 transgenic lines against wild type::GUS. Dry matter content (DMC) is given as % DMC from mean of three replicates. ***: statistically significant at P value < 0.001.

Unfortunately, the roots did not develop fully in small pots (1L). The physical root structure made the harvest and selection of appropriate roots for PPD assessment difficult because some roots had to be broken to detach them from the main plant (Figure 7.10). Luckily, since the plants performed well,

we were able to collect sufficient roots sample for PPD assessment.

Figure 7.10. Variation in root structure of wild type and BGLU23 expressing-transgenic cassava after 6 months of planting. Three plants were harvested and were used as one biological replicate for PPD assessment.

As mentioned earlier, we observed one distinctive transgenic line showing very significant differences in some phenotypic traits such as plant growth and morphology compared to wild type or other transgenic lines. The differences were more obvious when it was grown in the bigger pot (10-L) (Figure 7.11). This line, PAT::BGLU23-expresing line 3, showed the lowest growth rate, dwarf effect, stem and leaf morphology variations.

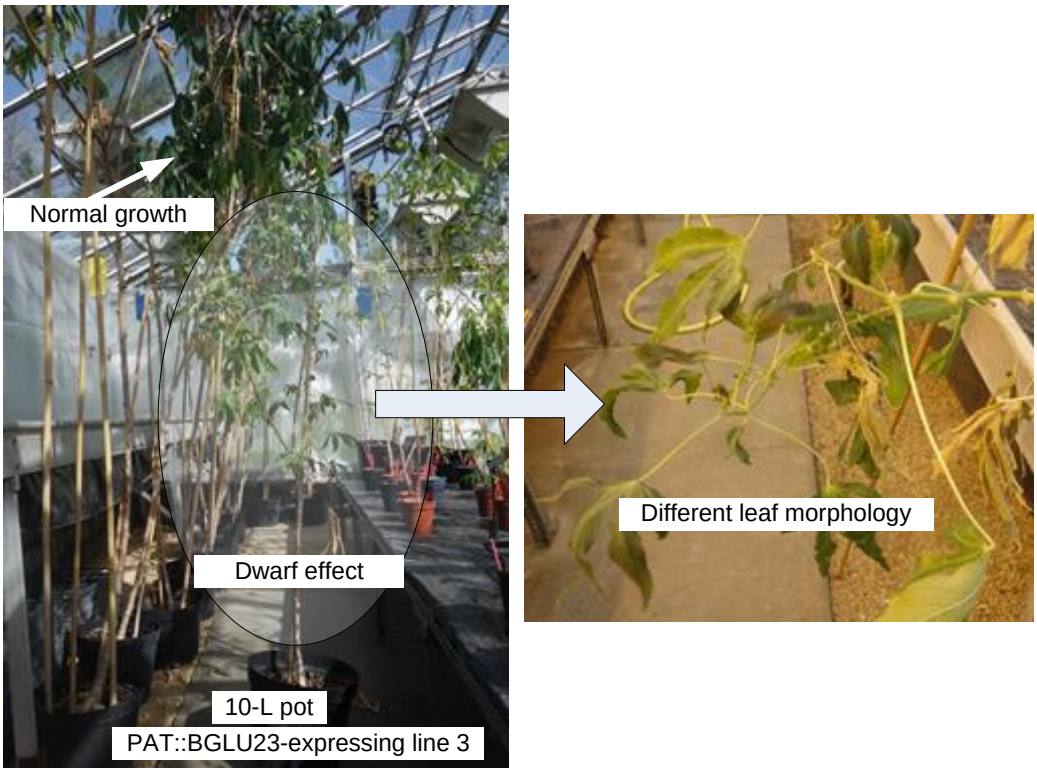


Figure 7.11. Dwarf effect and leaf morphology variation in PAT::BGLU23-expressing line 3 after 12 months. The plants were grown in 10 L pot where they could grow normal as in the field. Normal growth plant was shown in white arrow on the left.

7.3.2. Effects of overexpression of BGLU23 on cassava PPD

7.3.2.1. Overexpression of BGLU23 in cassava reduces PPD

Cassava roots with diameter size between 15-30 mm from 6-month-old plants were selected for PPD assessment in order to minimise standard error due to root size differences. The results presented in figure 7.12 revealed that two BGLU23-expressing transgenic cassava lines showed a highly significant (P value < 0.01) decreased in % discoloration ranged between 14% and 17% compared to wild type::GUS (43%) at day 6 and the two lines showed a statistically significant different at P value < 0.05. In this study, the use of root-specific PATATIN promoter showed significant difference in the PPD assessment results compared to constitutive CaMV-35S promoter.

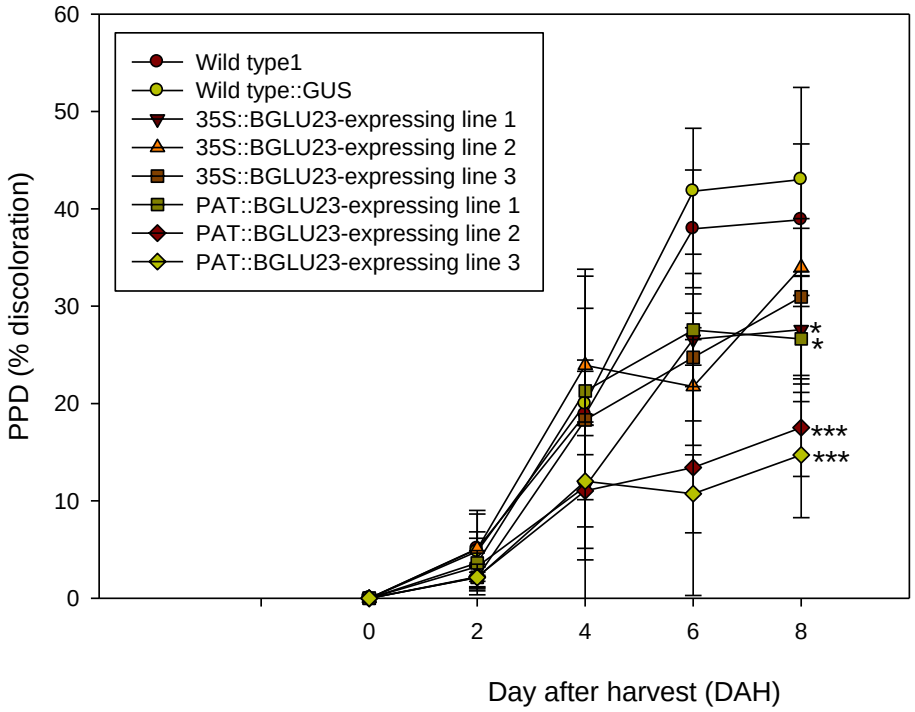


Figure 7.12. PPD assessment of BGLU23-expressing transgenic lines against wild type at 2, 4, 6 and 8 days after harvest. All transgenic lines showed a significant decrease in % discoloration compared to wild type. PPD is given as average of % discoloration (PPD score x 100) ± SD. % discoloration of root is normalised to % discoloration at time 0. SD refers to the standard deviation of three biological replicates. **: significant at P value < 0.01, ***: highly significant at P value < 0.001.

PPD rate (day^{-1}) and extrapolation of delayed PPD time

PPD rate (day^{-1}) was calculated according to Moyib et al (2015). The PPD rate (day^{-1}) of the BGLU23-expressing transgenic lines varied between 11% and 25% day^{-1}. Using formula in section 2.12.9.3, an estimated delayed PPD time in transgenic lines was speculatively extrapolated to be between 1 and 3 days (Table 7.1). Compared to *MeSGT*1-knock down lines, BGLU23-expressing transgenic lines proved less effective in delaying PPD. However, as also mentioned in chapter 6, this is an extrapolation of past % discoloration trends and assumes that the PPD rate remained constant. Therefore, more studies such as extended PPD induction up to 2-3 weeks would be necessary to test this prediction.

Table 7.1. Analysis of the PPD rate (day^{-1}) and estimated delayed PPD time (day) in BGLU23-expressing transgenic lines.

No	Line	PPD rate (day^{-1})	Estimated delayed PPD (day)
1	Wild type::GUS	34.21	0.00
2	35S::BGLU23 expressing line 1	21.63	0.70
3	35S::BGLU23 expressing line 2	25.40	0.79
4	35S::BGLU23 expressing line 3	23.67	0.72
5	PAT::BGLU23 expressing line 1	23.53	0.61
6	PAT::BGLU23 expressing line 2	13.46	2.11
7	PAT::BGLU23 expressing line 3	11.72	2.65

Root images showed less % discoloration in roots of BGLU23-expressing transgenic lines

Figure 7.13 shows substantial reduction in root discoloration of BGLU23-expressing transgenic lines compared to wild type. In here, we gave an example of root discoloration appearances between wild type and the selected two transgenic lines with the lowest % root discoloration: PAT::BGLU23-expressing line 2 and PAT::BGLU23-expressing line 3.

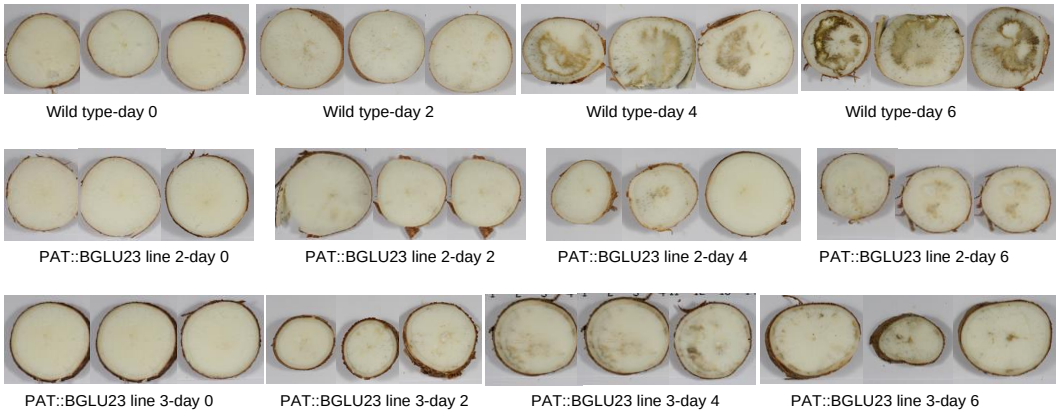


Figure 7.13. Root discoloration changes of the selected BGLU23-expressing transgenic lines over PPD time course. The diameter of sliced root was between 15 mm to 30 mm.

7.3.2. Overexpression of GBLU23 reduces scopoletin and scopolin in transgenic lines

Biochemical changes during PPD in both wild type controls and BGLU23-expressing transgenic lines were analysed to investigate further the effect of overexpression of BGLU23 in cassava. We measured scopoletin, scopolin, esculetin and esculin content in wild type and transgenic lines by LCMS following protocol in section 2.12.11. Of six BGLU23-expressing transgenic cassava lines, we only selected two best lines based on the lowest % root discoloration (Table 7.1): PAT::BGLU23-expressing line 2 (13.42 ± 13.14) and PAT::BGLU23-expressing line 3 (10.72 ± 4.01). Quantification by LCMS presented in figure 7.14 reveals that overexpression of BGLU23 in cassava reduced scopoletin and scopolin in the fresh root at day 0 and at day 6. There was a fluctuation in scopoletin and scopolin accumulation between those periods, where they increased and reached the peak at different time points. Interestingly, BGLU23-expressing transgenic lines exposed similar trends to *MeSGT*1-RNAi transgenic lines in scopoletin and scopolin accumulation during PPD onset.

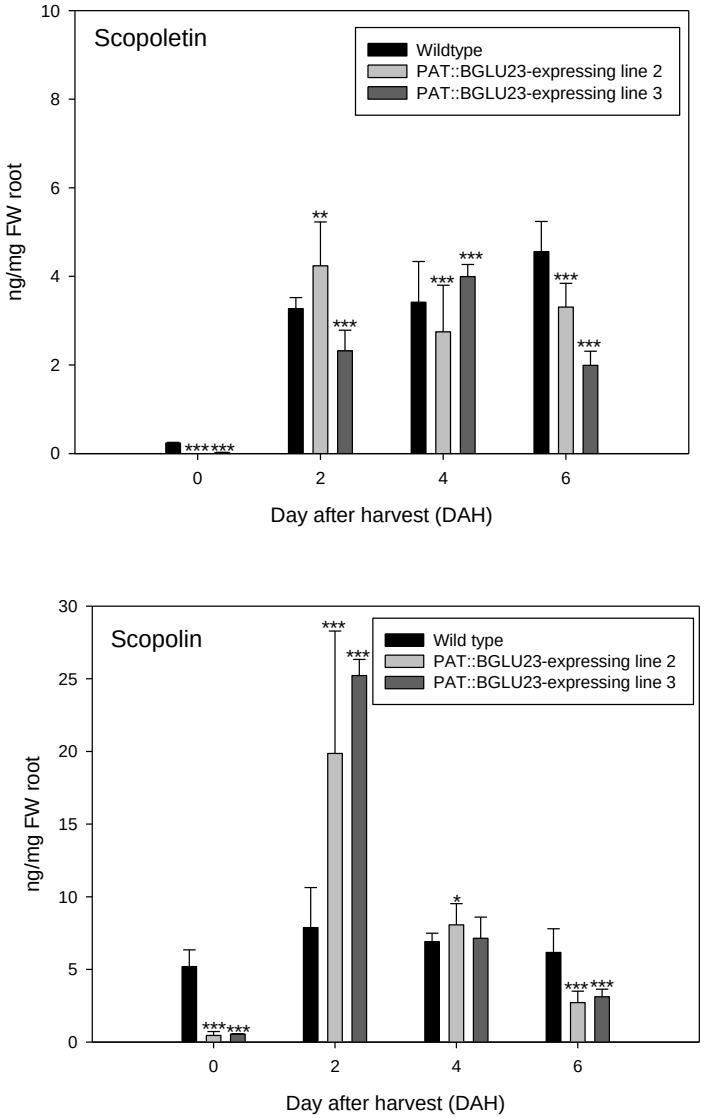


Figure 7.14. Changes of scopoletin and scopolin accumulation during PPD progress in BGLU23-expressing transgenic lines compared to wild type. The transgenic lines showed a significant reduction in scopoletin and scopolin accumulation at day 6 compared to wild type. The value is given as average of scopoletin or scopolin content ± SD (ng/mg FW root). SD refers to the standard deviation of three biological replicates. *: significant different to wild type at P value < 0.05, **: highly significant different at P value < 0.01 and ***: highly significant (P value < 0.001).

Scopoletin accumulation in the two BGLU23-expressing transgenic lines increased and reached a peak at between day 2 and 4 and then decreased after day 4, while in wild type, it kept increasing until day 6. In PAT::BGLU23-

expressing line 2, scopoletin accumulation reached the peak of 4.23 ± 0.99 ng/mg fresh weight (FW) root at day 2, and 3.99 ± 0.27 ng/mg FW root in PAT::BGLU23-expressing line 3 at day 4. Scopolin accumulation in roots of the two transgenic lines was significantly lower than wild type in the fresh roots (day 0). Surprisingly, scopolin accumulation in the transgenic lines increased dramatically at day 2 and reached the peak of 19.86 ± 8.42 ng/mg FW root in PAT::BGLU23-expressing line 2, and 25.21 ± 1.11 ng/mg FW root in the other line, which were highly significant higher than wild type (7.87 ± 2.75 ng/mg FW root). Compared with *MeSGT*1 RNAi transgenic lines, BGLU23-expressing transgenic lines even accumulated higher scopolin at the same period of day 2.

In addition, we also detected trace amount of esculetin and its glucoside, esculin in the roots of wild type and transgenic lines during PPD. Overall, the levels of esculetin and esculin in the two transgenic lines were significantly lower than that of the wild type (Figure 7.15). In wild type, esculetin increased and reach the peak at day 4, while esculin kept increasing over PPD time until its levels was at similar level to scopolin at day 6, which was relatively high. In BGLU23-expressing transgenic lines, esculetin showed the same trends as of *MeSGT*1-RNAi transgenic lines where it increased over PPD time course and reached the maximum levels at between day 2 and 4. Esculin, on the other hand, showed a slight increase until day 6.

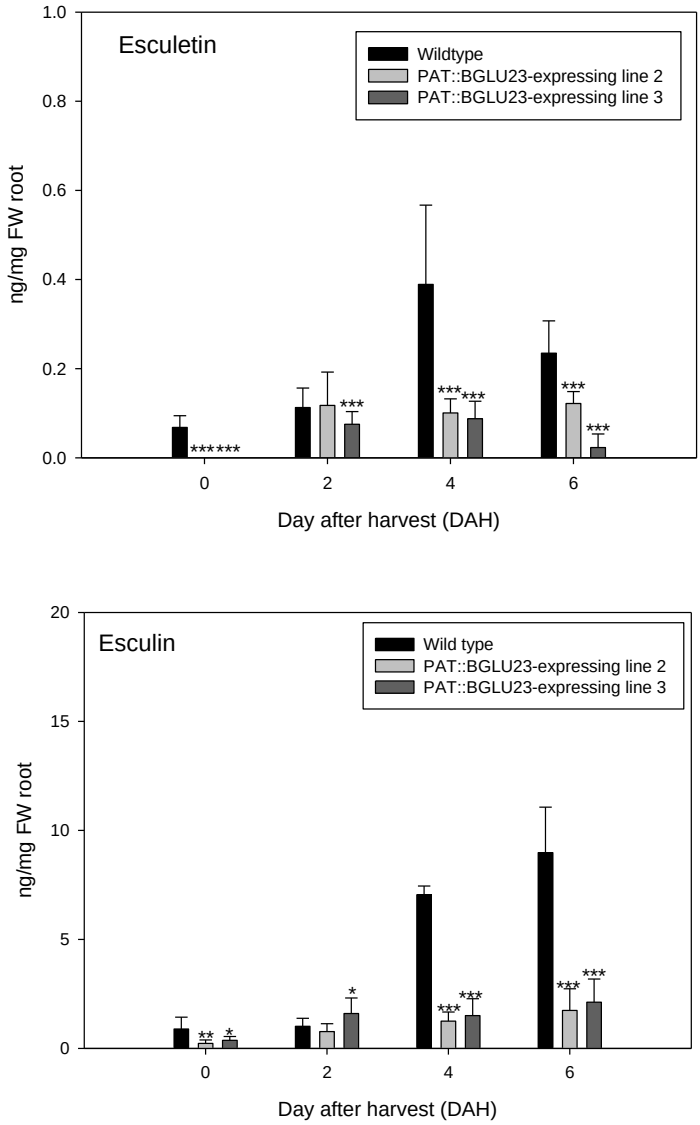


Figure 7.15. Changes of esculetin and esculin accumulation during PPD progress in BGLU23-expressing transgenic lines against wild type. The transgenic lines showed a significant reduction in scopoletin and scopolin accumulation at day 6 compared to wild type. The value is given as average of esculetin or esculin content ± SD (ng/mg FW root). SD refers to the standard deviation of three biological replicates. ***: highly significant (P value < 0.001).

7.3.3. Scopoletin accumulation showed positive correlation with PPD

Correlation analysis between scopoletin/scopolin and PPD response was done by plotting scopoletin/scopolin content with % discoloration (Figure 7.16 and Figure 7.17). Scopoletin showed a positive correlation with PPD

progress (% discoloration), while scopolin showed negative correlation.

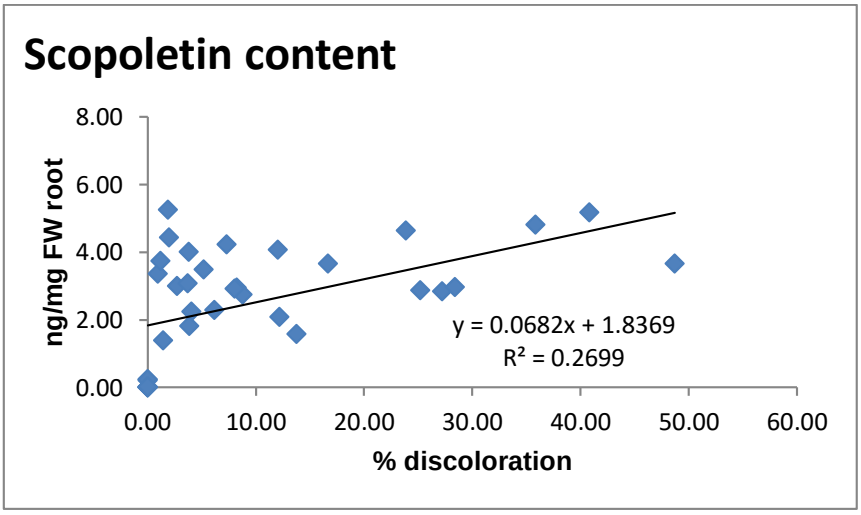


Figure 7.16. Correlation analysis between scopoletin content and PPD. Scopoletin is strongly correlated to PPD (R^2 = 0.2699). Data presented are average of three biological replicates data over time course of PPD (day 0, 2, 4 and 6).

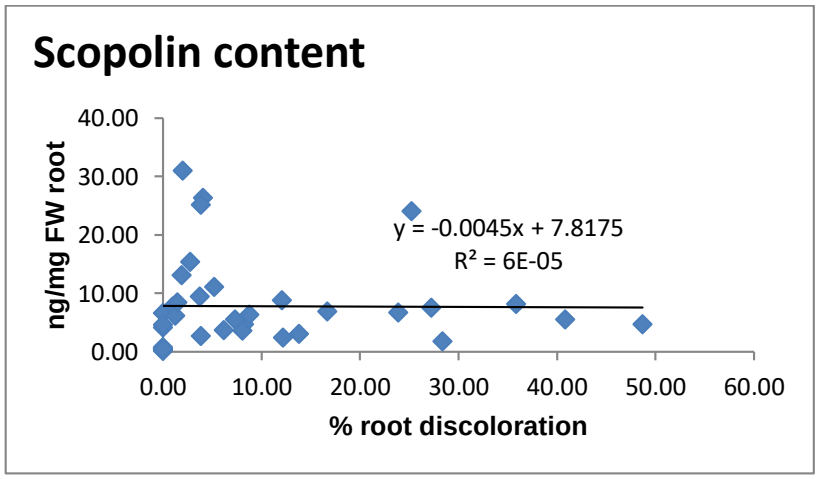


Figure 7.17. Correlation analysis between scopolin content and PPD. Scopolin shows weak correlation to PPD (R^2 = 6E-05). Data presented are average of three biological replicates data over time course of PPD (day 0, 2, 4 and 6).

7.3.4. Changes of BGLU23 gene expression levels during PPD

7.3.4.1. Verification of primer efficiency for qRT-PCR

The expression of BGLU23 in cassava during PPD was analysed by qRT-PCR using comparative C_T experiment with cDNA from roots. Before

running comparative C_T experiment, we first designed three primers pairs with estimated PCR products ranging from 64 bp to 89 bp and tested their amplification efficiency using relative standard curve experiment. To create standard curve, C_T values were plotted against quantity of cDNA (Figure 7.18). The average amplification efficiency varied among three primers pairs (Table 7.2). Primer 3 showed best efficiency of 101.02 %, therefore it was then used for comparative C_T experiment.

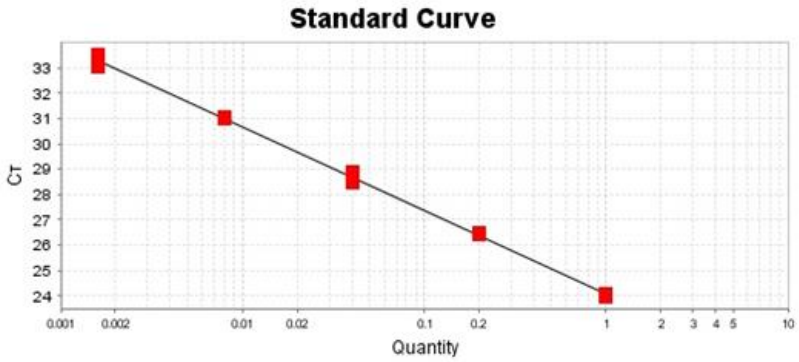


Figure 7.18. Standard curve of BGLU23 obtained from primer 2 with best efficiency value of 101.02 %.

Table 7.2. Summary of relative standard curve experiment.

No	Primer	Slope	R^2	Efficiency %
1	Reference gene:Ubq10	-3.409	0.996	96.498
2	BGLU23 Primer 1	-3.724	0.995	85.573
3	BGLU23 Primer 2	-3.442	0.988	95.241
4	BGLU23 Primer 3	-3.298	0.998	101.025

7.3.4. Expression profiling (C_T values) of BGLU23 gene during PPD

The expression levels of BGLU23 in wild type and transgenic cassava lines were given as C_T values. Figure 7.19 reveals that the target mRNA was hardly detected or not expressed in wild type cassava (C_T value >30) because this gene is a foreign gene for cassava.

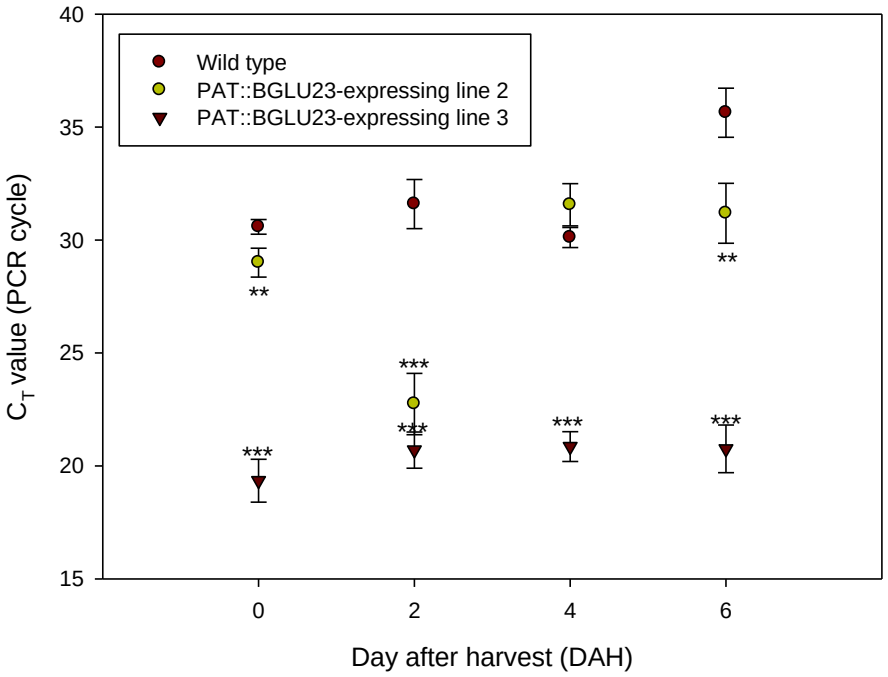


Figure 7.19. Expression level of BGLU23 gene in root samples of BGLU23-expressing transgenic cassava lines and wild type during PPD progress. BGLU23 gene is upregulated at day 2 in all samples. Data given is an average of C_T values ± SD. SD refers to standard deviation of three biological replicates and each run with three technical replicates.

Interestingly, two BGLU23-expressing transgenic cassava lines exhibited contrasting gene expression profiles. In PAT::BGLU23-expressing line 2 , BGLU23 was expressed at a low level in fresh root (day 0) and it increased at day 2. While, in PAT::BGLU23-expressing line 3, the BGLU23 was highly significantly (P value < 0.001) expressed in fresh roots from the beginning at day 0 and its expression levels remained steady high during PPD progress until day 6. The latter line was found to have distinct plant morphological characters than the other lines (section 7.3.1). The pattern of C_T values during PPD time course showed that the BGLU23 was significantly highly up-regulated at day 2 in the two transgenic lines, although in one line, its expression levels were considerably high at all times.

7.4. Discussion

This chapter aimed to examine the effects of overexpression of Arabidopsis scopolin-BG (BGLU23) in cassava on scopoletin and scopolin accumulation and PPD response in the storage roots. Coupled with the previous results in chapter 6, information from this chapter would complete our studies on investigating the role of the interconversion of scopoletin and scopolin in cassava PPD. This would add more to our understanding of PPD mechanisms and may suggest another strategy to delay PPD progress in cassava in addition to the one in chapter 6. BGLU23 is a beta-glucosidase from Arabidopsis that had shown a high and specific activity towards scopolin (Ahn et al., 2010). Overexpressing BGLU23 in cassava could potentially accelerate the de-glucosylation of scopolin to release scopoletin in response to such a stress condition like wounding. PPD is considered as an active physiologically and biochemically process that has strong parallels to wound responses. Wounding inevitably occurs during harvest and handling, which triggers an oxidative burst followed by changes in gene expression, protein synthesis and accumulation of various defensive compounds (Buschmann et al., 2000, Huang et al., 2001, Reilly et al., 2003, Reilly et al., 2007, Iyer et al., 2010, Beeching et al., 1998, Owiti et al., 2011).

The present study reveals that overexpression of BGLU23 in cassava reduced not only scopolin, but also scopoletin accumulation in the fresh roots and deteriorating roots at day 6, also it delayed PPD in transgenic cassava compared to wild type. Four coumarins including scopoletin, scopolin, esculetin and esculin were either undetectable or present at very low levels in the fresh roots. These findings are consistent with those of (Bayoumi et al., 2010, Buschmann et al., 2000, Tanaka et al., 1983). In the fresh roots, scopoletin and esculetin were detected at trace amounts, suggesting that these active compounds cannot accumulate to high level in normal condition and those are most likely to be converted into their glycosides: scopolin and esculin via glycosylation. The latter two glucosides

were detected at higher levels than their active form: scopoletin and esculetin, confirming that those are stored as inactive form in the cells. However, compared to other plant species such as Arabidopsis (Kai et al., 2008, Kai et al., 2006) and tobacco (Chong et al., 2002), scopolin in cassava fresh roots was significantly lower. This result is consistent with our previous results (chapter 6) and other studies on cassava (Bayoumi et al., 2010, Buschmann et al., 2000, Tanaka et al., 1983).

We found that four coumarins including scopoletin, scopolin, esculetin and esculin accumulated during PPD development. In transgenic lines, there was a significant increase not only in scopoletin but also scopolin accumulation between day 2 and day 4 and then decrease afterwards. A slight increase was shown by esculetin and esculin at those periods. In wild type, on the other hand, scopoletin and esculin consistently increase until day 6, while scopolin and esculetin reached the peak at day 2 and day 4, respectively. This demonstrates that those compounds play key roles in defense mechanisms under stress conditions such as wounding in cassava. Similar to the *MeSGT*1 RNAi transgenic lines (chapter 6), the two BGLU23-expressing lines also showed different peak times of scopoletin and scopolin accumulation between day 2 and day 4. The accumulation and fluctuation of those compounds during PPD might be simply reflecting the cassava response towards wounding. Moreover, an increase in scopolin accumulation in both wild type and transgenic lines at day 2 may suggest that a) scopolin is also biosynthesised *de novo* along with scopoletin towards wounding and b) the de-glucosylation of scopolin seems to play minor role in the production of scopoletin although the conversion of scopolin to scopoletin may have occurred as the BGLU23 was up-regulated at that period. Therefore, in terms of scopoletin, it is most likely to be biosynthesised *de novo* through phenylpropanoid metabolism rather than generated from the conversion of its glucoside, scopolin. In this chapter, scopoletin also showed positive correlation with PPD response in cassava,

confirming that scopoletin plays a dominant role in this process, which consistent with our previous study (chapter 6) and other studies (Liu et al., 2017, Uarrota et al., 2015, Sánchez et al., 2013, Bayoumi et al., 2010, Buschmann et al., 2000, Wheatley and Schwabe, 1985, Tanaka et al., 1983). In addition, we found dry matter content (DMC) showed negative correlation with PPD, which is contrary with other studies (Wenham, 1995, Chavez et al., 2005).

Overall, this study has demonstrated that overexpression of scopolin-BG in cassava also led to the decreased scopoletin and scopolin and delayed PPD. However, the de-glucosylation of scopolin does not seem to play a substantial role in the production of scopoletin in response to wounding in cassava. In fact, some BGLU23-expressing transgenic cassava lines showed a significant reduction in scopoletin and scopolin content at day 6 and delayed PPD, more studies on the de-glucosylation step in cassava might be needed. Although the significance of this finding is clear, the first limitation of this study is that we were not able to identify specific beta-glucosidase for scopolin in the cassava genome for gene silencing. Therefore, for future work, it is still interesting to identify best candidate cassava gene encoding scopolin-BG from an updated cassava genome database on Phytozome for gene silencing study and investigate how the inhibition of the de-glucosylation of scopolin will affect scopoletin and scopolin accumulation and cassava PPD response.

Chapter 8. General Discussion

Chapter 8. General Discussion

This chapter summarises all the results, critically evaluates them in terms of the literature and outlines directions for future work.

8.1. Summary of the thesis

The thesis has focused on identifying and characterising the genes responsible for the interconversion of scopoletin and scopolin in cassava and investigating their roles in cassava postharvest physiological deterioration (PPD). Chapters 3 and 4 revealed the diversity and function of scopoletin-glucosyltransferase (scopoletin-GT) and scopolin-beta-glucosidase (scopolin-BG) gene-families in cassava. We found that the genes for both enzymes are members of super-families that contain up to hundreds of genes in the cassava genome. We identified six out of 164 cassava genes encoding scopoletin-GT that are most closely related to the reference gene, *togt*1, but only two were expressed in the roots, one of which was therefore used in the RNAi gene silencing study. Unfortunately, none of cassava genes encoding scopolin-BG proved to be sufficiently closely related to the reference gene, BGLU23, to be accepted with confidence for parallel further analysis. Therefore, in the case of scopolin-BG, it was decided to overexpress, rather than knock down, scopolin-BG from Arabidopsis, BGLU23, in cassava instead. The selected genes: *MeSGT*1 for scopoletin-GT and BGLU23 for scopolin-BG, were used to create RNAi and overexpression constructs, respectively for cassava transformation (Chapter 5). Transformation successfully generated 14 independent transgenic cassava lines for both scopoletin-GT (*MeSGT*1) RNAi-mediated gene knock-down and scopolin-BG (BGLU23)-expressing lines. Those were grown under glasshouse conditions at the University of Bath for PPD and other assessments discussed in chapter 6 and chapter 7, respectively.

Chapters 6 and 7 examined the effects of down regulation of cassava

scopoletin-GT (*MeSGT*1) via RNAi gene silencing and overexpression of BGLU23 in cassava on scopoletin and scopolin accumulation and PPD response in transgenic cassava. This revealed that both strategies led to the decrease of scopoletin and scopolin content and significant reduction of % root discoloration (PPD) of the transgenic cassava. All types of transgenic cassava lines: *MeSGT*1 RNAi-silencing and BGLU23 expressing-lines showed scopoletin and scopolin alteration and accumulation in the fresh root and over the PPD time course. A positive correlation between scopoletin accumulation and % discoloration (PPD) confirms the importance of scopoletin in the root discoloration response.

8.2. Discussion

8.2.1. Scopoletin-glucosyltransferase and scopolin-beta-glucosidase from cassava

Glucosyltransferase (GT) and beta-glucosidase (BG) enzymes are commonly found in many plant species. Although information on the gene families for those two enzymes in cassava is currently available in the PlantCAZyme database (Ekstrom et al., 2014), no studies on the specific genes for scopoletin-GT and scopolin-BG in cassava have been reported. The first finding of this study, therefore, will facilitate our understanding of the identity, diversity and function of the selected cassava genes encoding scopoletin-GT and scopolin-BG. Moreover, identifying the specific genes and confirming their function are fundamental to minimise off-target effects in the genetic modification. Like in other plant species, we found that scopoletin-GT and scopolin-BG enzymes are encoded by multiple genes, which were distributed across different regions or chromosomes in the cassava genome (Chapter 3). Although, it is not clear whether this encoding is correlated with a wide range of enzyme structures in metabolic compartments or it is simply for rapid production of the enzymes under stress conditions (Dixon and Paiva, 1995), this large number of genes is more likely to be linked to the diversity of the substrates on which they act.

Glucosyltransferases (GTs) are a highly divergent, polyphyletic, and multigene family (Mackenzie et al., 1997). Some plant GT gene families have been classified and information on them is accessible in the Carbohydrate-Active enZYmes (CAZY) database- (Davies et al., 2005) and in the specific Plant Carbohydrate-Active enZyme (PlantCAZyme) database (Ekstrom et al., 2014). According to the latter database, 481 GT genes in cassava have been identified and classified into 41 gene families (Chapter 3). We identified the selected 22 cassava scopoletin-GT-like genes that are closely related to the reference gene, *togt*1, as belong to glucosyltransferase family-1 (GT-1). The diversity of scopoletin-GT-like genes in cassava may be correlated with a broad enzyme activity, as was shown by two UDP-glucosyltransferases from cassava, UGT85K4 and UGT85K5, catalysing the last step in the biosynthesis of cyanogenic glucoside (Kannangara et al., 2011). This broad enzyme activity indicates that substrate specificities of some UDP-glucosyltransferases are defined by region-specific or region-selective features of the aglycones (Vogt and Jones, 2000). However, it needs to be analysed further to confirm whether the broad substrate specificity shown by those two glucosyltransferases also reflects the specificity of scopoletin-glucosyltransferases identified in this study.

UDP-glucosyltransferases (UGTs) regulates glycosylation steps in general phenylpropanoid metabolism that plays an important role in defence response and stress tolerance (Bowles et al., 2005, Vogt and Jones, 2000). Glycosylation has the ability to change phenylpropanoid solubility, stability, toxic potential, as well as influence compartmentalisation and biological activity (Le Roy et al., 2016). This reaction takes place in the cytosol before the products (glycosides) are transported to and stored in the vacuole (Vogt and Jones, 2000). The importance of glycosylation in the stabilisation of secondary metabolites has been shown by different members of glycoside family including anthocyanins, the glucosinolates and cyanogenic

glycosides (Kahn et al., 1997, Vogt and Jones, 2000). Scopoletin (6-methoxy-7-hydroxycoumarin) is a highly reactive phenolic hydroxyl and is a known reactant of peroxidases *in vitro* (Marquez and Dunford, 1995, Chong et al., 2002). Therefore, glucosylation of scopoletin to scopolin under normal conditions by scopoletin-glucosyltransferases (scopoletin-GTs) is believed to protect scopoletin against cellular oxidases as well as improve its solubility in the cell (Chong et al., 1999).

The function of the selected cassava gene for scopoletin-GT was confirmed using an Arabidopsis mutant, which as discussed in chapter 4. According to qRT-PCR data in chapter 6, the gene for this enzyme, *MeSGT*1, is highly expressed, which clearly indicates the occurrence of glucosylation of scopoletin. Under stress conditions such as the artificial wounding used in this study, the gene encoding scopoletin-GT was up- regulated at day 4. The up-regulation of this gene over the PPD period is in agreement with several studies (Owiti et al., 2011, Reilly et al., 2007). It demonstrates that scopolin may be produced in response to wounding as an antibiotic compound against microbial invasion following the first type of deterioration (PPD) that usually occurs after 4 days of harvesting. Scopolin has toxic effects on the fungus *Sclerotinia sclerotiorum* (Prats et al., 2006).

Beta-glucosidases (E.C.3.2.1.21) belong to the glycoside hydrolase (GH) family that is widely distributed in all domains of living organisms (Ahn et al., 2010). In plants, beta-glucosidases play roles in lignification, catabolism of cell wall oligosaccharides, defense, phytohormone conjugate activation, and scent release, as well as in both sides of plant–microbe and plant–insect interactions. The roles of these enzymes are supposed to be controlled by their substrate-specificities, their tissue and subcellular localisation, and the conditions under which they are exposed to their physiological substrates (Cairns and Esen, 2010). Like the previous enzyme, we also found that scopolin-beta-glucosidase (scopolin-BG) in

cassava was also encoded by multiple genes that were identified on different locations of the genome (Chapter 3). Identified cassava scopolin-BG genes were widely distributed in the phylogenetic tree and none of them were closely related to the reference gene, BGLU23, which prevented identifying homologous cassava genes with specific activity towards scopolin with any confidence. Because there were no significant changes in scopoletin and scopolin content in both the Arabidopsis knock-out line and *MeSBG*4-expressing Arabidopsis transgenic line, we could not determine the function of the selected cassava scopolin-BG, *MeSBG*4. Therefore, the function of this gene remained unclear. While we could have expressed the selected cassava gene in bacteria cells and determined its substrate specificity this was not attempted as scopolin was not commercially available and needed to be isolated from plant sources, which might have been a limitation of using scopolin in the experiment. However, scopolin is now commercially available but very expensive, which could permit such test in the future.

In the biosynthesis of scopoletin, this enzyme responsible for the de-glucosylation of scopolin to release scopoletin in response to stress conditions such wounding or microbial infection. Beta-glucosidase also has been known as detonators for plant chemical defense (Morant et al., 2008), which means this enzyme is mostly activated in response to either biotic or abiotic stresses. Analysis of qRT-PCR showed that the BGLU23 was significantly up-regulated at day 2 after harvest, which was accompanied by a dramatic increase in scopoletin levels (Chapter 7). This indicates that the BGLU23 is wound-induced gene to release scopoletin.

Cassava scopolin-beta-glucosidase genes are still not well characterised yet. The most studied beta-glucosidase from cassava is cyanide-beta-glucosidase (linamarase). Expression of the gene for this enzyme in *P. pastoris* revealed that it has a great ability to transfer glucose (Guo et al.,

2015). In plants, antibiotic classification is based on how they are produced; cyanogenic glucosides are considered as phytoanticipins (VanEtten et al., 1994, Moller, 2010). This compound is activated by the action of beta-glucosidase to release toxic molecules in response to pathogen attack (Moller, 2010). Similarly, the scopoletin glucoside, scopolin, may also be considered as belonging to this plant antibiotic class. Beta glucosidases are localised in a different compartment from the substrate (Morant et al., 2008). In cassava, while beta-glucosides such as cyanogenic glucoside and scopoletin-glucoside, scopolin, are stored in vacuole, beta-glucosidases are localised in apoplast. Upon tissue disruption, beta-glucosidases react with cyanogenic glucoside to release cyanide (Moller, 2010) as scopolin is broken down to scopoletin by the action of scopolin-beta-glucosidase.

8.2.2. The role of interconversion of scopoletin and scopolin in cassava PPD

PPD is a physiologically and biochemically complex processes involving many reactions including oxidative burst, altered gene expression and protein synthesis as well as production of various secondary metabolites (Reilly et al., 2003, Reilly et al., 2007, Owiti et al., 2011, Vanderschuren et al., 2014, Buschmann et al., 2000, Bayoumi et al., 2010, Iyer et al., 2010). Our results confirm changes in physiological, biochemical and gene expression of the cassava roots during PPD development, which reflects its complexity. In the past 20 years, there have been significant increases in -omic studies including transcriptomic, proteomic and metabolomics of the cassava storage roots in order to understand better PPD mechanisms. PPD has been considered as a product of an oxidative process (Salcedo and Siritunga, 2011, Reilly et al., 2003). Oxidative burst is recognised as an early plant response to any stress conditions or perceptions such as injury or wounding and pathogen attack (Wojtaszek, 1997). In cassava, this event was detected within the first 15 min upon tissue disruption, followed by the altered gene regulation mostly related to the modulation of reactive oxygen

species (ROS), particularly by the enzymes catalase and peroxidase, and the production of secondary metabolites (Reilly et al., 2003). The rapid production of ROS or oxidative burst is associated with cyanogenesis that occurs immediately after tissue disruption. Cyanogenesis inhibits complex IV in mitochondrial electron transfer chain and blocks the mitochondrial respiration. This inhibition causes a rapid and massive production of ROS at complex I and III which triggers PPD (Zidenga et al., 2012). The visual symptom of PPD, blue-black discoloration of the roots, is usually accompanied by the accumulation of fluorescence compounds, which have been identified as coumarins including scopoletin, scopolin, esculetin and esculin (Buschmann et al., 2000).

In terms of blue-black discoloration of the roots, scopoletin plays the most important role compared to other coumarins. Oxidation of scopoletin by reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) generated in the early events of PPD resulted in a blue-black colour in the root, visually observed as PPD. There is a strong relationship between ROS and the accumulation of coumarins, particularly scopoletin although various events such as cell wall remodelling, programmed cell death (PCD), starch and lipid degradation, are also taking place during PPD development. Thus, modifying these two major components, ROS and scopoletin, in the cassava roots could be the key to delay blue-black discoloration development. Most efforts so far have focused on ROS manipulation by reducing ROS production or enhancing ROS scavenging (Zidenga et al., 2012, Xu et al., 2013). However, Liu et al (2017) were the first to modify scopoletin content in cassava roots by inhibiting the gene encoding feruloyl CoA 6′-hydroxylase responsible for the biosynthesis of scopoletin in the ferulate pathway using RNAi silencing. All these strategies have proven to delay cassava PPD effectively.

Since the status of scopoletin is also determined by the interconversion of

scopoletin and scopolin, our findings should help fill the gap in this area by improving our understanding of the biochemical pathway related with PPD. The two strategies from this study: knocking down cassava scopoletin-GT, *MeSGT*1, and overexpressing Arabidopsis scopolin-BG, BGLU23, have shown to significantly reduce scopoletin and scopolin content in the fresh cassava roots and deteriorating roots at day 6 resulted in longer delayed PPD than wild type control. The decreased scopoletin levels in *MeSGT*1-RNAi expressing transgenic in the fresh roots is comparable to the result from tobacco (Chong et al., 2002), although it was unexpected. The inhibition of the glucosylation of scopoletin by down regulating scopoletin-GT in cassava would potentially increase scopoletin accumulation in the cells. The latter compound might have been increased; however, it is well-known that scopoletin is very reactive and is a reactant of peroxidases *in vitro* and *in vivo* (Chong et al., 1999). Therefore, compromising the accumulation of scopoletin could enhance peroxidase activity resulted in the higher oxidation rate of scopoletin (Chong et al., 2002). In wild type cassava, this gene (*MeSGT*1) was highly expressed in the fresh roots indicating that the gene is activated under normal cellular processes to glucosylate scopoletin to scopolin. Glucosylation seems to play important role in the status of both scopoletin and scopolin that affects PPD. All RNAi transgenic lines for this step were significantly lower in scopoletin and scopolin levels, and also delayed PPD, compared to wild type. Likewise, overexpressing BGLU23 would be able to accelerate the accumulation of scopoletin from scopolin. These transgenic lines demonstrated similar effects on the coumarins content and PPD response with the scopoletin-GT RNAi transgenic lines. If the BGLU23 was activated under normal conditions during cellular processes, it also could potentially trigger the higher activity of cellular peroxidase.

In response to wounding, cassava roots produced three main coumarins, scopoletin, scopolin and esculin, which is consistent with (Tanaka et al.,

1983) and trace amounts of esculetin (Buschmann et al., 2000, Bayoumi et al., 2010). However, among those four coumarins only scopoletin has been known to play a vital role in root discoloration by forming blue complex when it is oxidised (Miller et al., 1975). Wheatley (1985) observed more intense and rapid discoloration when exogenous scopoletin was applied onto the fresh cassava root, suggesting the direct and central role of scopoletin in root discoloration or PPD. During the PPD time course, scopoletin was found to dramatically increase at an early stage of PPD, day 2, then gradually decrease, although some lines demonstrated a later peak time at day 4 (Chapters 6 and 7). The increased scopoletin is most likely due to the activity of PAL and scopolin-beta-glucosidase (scopolin-BG) enzymes. PAL is the first key enzyme in the phenylpropanoid metabolism leading to the production of various secondary metabolites including scopoletin and scopolin. This enzyme is induced shortly after injury and usually show peak activity at 36-72 h (Beeching et al., 1994, Beeching et al., 2000). The increased activity of PAL is also observed in other wounded crops such as sweet potato, yam and potato, demonstrating that cassava shows some commonality in wound response with those crops (Beeching et al., 1998). This enzyme has the ability to break down glucose molecule from scopolin to release scopoletin.

The increased scopoletin after day 2 while the BGLU23 was less expressed suggests that scopoletin is mainly produced *de novo* from the phenylpropanoid pathway rather than from the de-glucosylation of scopolin. Another possibility is that cassava scopolin-BG genes that have not been identified or tested in this study might have been activated more than BGLU23 to release scopoletin. Interestingly, during the same period of day 2, scopolin levels also increased showing that the glucosylation of scopoletin is also activated. Moreover, no significant reduction of scopolin levels over the PPD time-course assayed after day 2 could support the hypothesis that the de-glucosylation of scopolin may not play crucial role in

the accumulation of scopoletin that contributes to PPD in cassava. Therefore, identifying the genes that have specific activity leading to the biosynthesis of scopoletin would be the key to reducing scopoletin accumulation. Knocking down the genes for a key enzyme of feruloyl CoA 6' Hydroxylase in the biosynthesis of scopoletin considerably reduced scopoletin accumulation in the cassava roots (Liu et al., 2017). However, this approach had shown phenotypic changes under glasshouse study although it needs to be confirmed under field trial. On the other hand, all transgenic lines from this study showed normal growth compared to the control wild type except one line of BGLU23-expressing line (Chapter 7). Therefore, our approaches could be alternative ways to tackle the PPD.

In terms of scopolin accumulation, cassava showed different behaviour compared to other plant species. In fresh cassava roots, scopolin content was significantly lower than in Arabidopsis (Kai et al., 2008, Kai et al., 2006) and tobacco (Chong et al., 2002). In cassava, scopolin seems to play a role in another defence mechanism against microbial attack rather than as a phytoanticipin compound to release scopoletin. Scopolin has been known to possess fungi toxic activity towards *Sclerotinia sclerotiorum* (Prats et al., 2006). A dramatic increase in scopolin accumulation over the PPD period, particularly at day 2, in all transgenic lines is more surprising since it was hypothesised to be decreased by either the inhibition of glucosylation via *MeSGT*1 knock down or the acceleration of de-glucosylation of scopolin via BGLU23 overexpression in cassava. These counter-intuitive findings suggest the need for further work to resolve the various contributions of the principal and alternative biosynthetic pathways to these coumarins. To date there is a little information available on scopolin analysis in cassava storage roots. Therefore, this study can be crucial since very few studies done on these four coumarins analysis in cassava roots for the past 30 years (Tanaka et al., 1983, Buschmann et al., 2000, Bayoumi et al., 2010). Most research on this area focused on only one coumarin, scopoletin (Liu et al.,

2017, Uarrota et al., 2015, Sánchez et al., 2013, Wheatley and Schwabe, 1985). Transcriptomic and metabolomics data found that the up-regulation of cassava scopoletin-GT, *MeSGT*1, and the accumulation of scopolin in wild type cassava showed different peak times. This data imply that the accumulation of transcript does not always correlate with the accumulation of the corresponding protein (Vanderschuren et al., 2014).

8.2.2. Limitation of the Current Study

Despite promising results, this study also has a number of limitations. The first limitation is that it was not possible to identify the best candidate cassava gene encoding scopolin-BG for RNAi gene silencing as planned in the beginning of the study. Second, we were not able to perform cassava transformation at Bath. Therefore, the transformation was done in collaboration with our collaborator at ETH Zurich. While this was a limitation in terms of the time and cost involved, it was also a benefit as we could take advantage of an established system and the research experience of other researchers. Third, the scopolin standard was not commercially available and it had to be isolated from cassava roots by HPLC, which was done by our colleague at Department of Pharmacy and Pharmacology, University of Bath. Thereby, delaying the availability of this coumarin. However, recently scopolin has now become commercially available, at a high price. The last, due to space limit in the glasshouse, we could only grow about 560 plants in 1-L pot, which restricts the number of lines and replicates tested.

8.3. Implications of the study & potential socio-economic impact

Our findings can contribute considerably to provide alternatives techniques to produce improved cassava lines with delayed PPD trait. Generating delayed PPD cassava lines would have a direct and significant socio-economic impact to the farmers, traders, processors and consumers. Due to massive potential losses, delayed PPD is one of the most important traits that needs to be addressed in order to maximise cassava's potential as a

food and industrial crop. Africa as the largest cassava producing continent is suffering most from this problem. It was estimated the physical losses due to PPD about 29% in Africa, while in Asia and in Latin America along with the Caribbean suffered less approximately 8% and 10%, respectively (Salcedo and Siritunga, 2011). In the recent study, the losses due to PPD was predicted to be nearly (1/3rd) one third of total cassava production world-wide (Saravan et al., 2016) although this sort of losses varied between countries (Naziri et al., 2014). In Nigeria, delaying PPD for several weeks would potentially avoid the economic losses due to PPD up to US$ 2.9 billion over a 20-year-period (Rudi et al., 2010). In the case of Northeast of Thailand, estimated benefits by delaying PPD up to 45 days about 26.5 million USD per year for starch industry and 8.5 million USD per year for farmers (Vlaar et al., 2007).

8.4. Future Work

Although the significance of this study in delaying cassava PPD is clear, more research is required to test several hypotheses generated in the discussion part. This section presents some potential directions for future work.

8.4.1. Investigating further on scopoletin and scopolin biosynthesis

During PPD, both scopoletin and scopolin levels increased. Scopoletin is most likely accumulated through *de novo* synthesis rather than de-glucosylation of scopolin. On the other hand, scopolin accumulation which was expected to decrease by the inhibition of glucosylation of scopoletin or acceleration of de-glucosylation of scopolin also increased. These findings suggest the need for further work to investigate the other alternative biosynthetic pathways to these coumarins.

8.4.2. Inhibiting ROS explosion and scopoletin formation

Testing double or triple constructs to prevent both ROS accumulation and

reduce scopoletin together in order to generate cassava lines with better delayed PPD response could be of interest.

8.4.3. Field study

In addition, it must be borne in mind that this study was conducted under glasshouse condition using small-pot (1-L) grown plants and a sliced root method for PPD assessment over a short period. Further research is, therefore needed to confirm the results in a real agronomic condition (field). Moreover, scopoletin with other coumarins are known to have anti-microbial activity against microbial or pathogen attack. Here is the biggest question remains to be resolved; how would the transgenic lines perform under real field condition? This will include the plant growth and development, PPD behaviour as well as their resistance to any microbial or pathogen attacks or diseases. We would like to emphasize that this future work is very important before generalised conclusions can be drawn.

8.5. Conclusions

The present study has added to our understanding of the biochemical and molecular basis for the post-harvest wound response of cassava storage roots. It also has provided alternative strategies to delay PPD progress through either RNAi-mediated cassava gene silencing of scopoletin-GT or overexpression of scopolin-BG from Arabidopsis-BGLU23 in cassava. Disrupting the interconversion of scopoletin and scopolin pathway led to the decrease of scopoletin and scopolin levels and delayed PPD in cassava. During PPD development, cassava roots produce three main coumarins: scopoletin, scopolin and esculin and a trace amount of esculetin. In addition, coupled with the previous findings in delaying PPD development by preventing ROS accumulation or reducing scopoletin content (Liu et al., 2017, Xu et al., 2013, Zidenga et al., 2012), this study has confirmed the importance of scopoletin in cassava PPD. Our findings can lead to future work to generate better delayed PPD cassava lines by combining the two

individual approaches, ROS prevention with scopoletin reduction using double or triple constructs.

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Appendices

Appendix I. Map of plasmids used in the gene construction

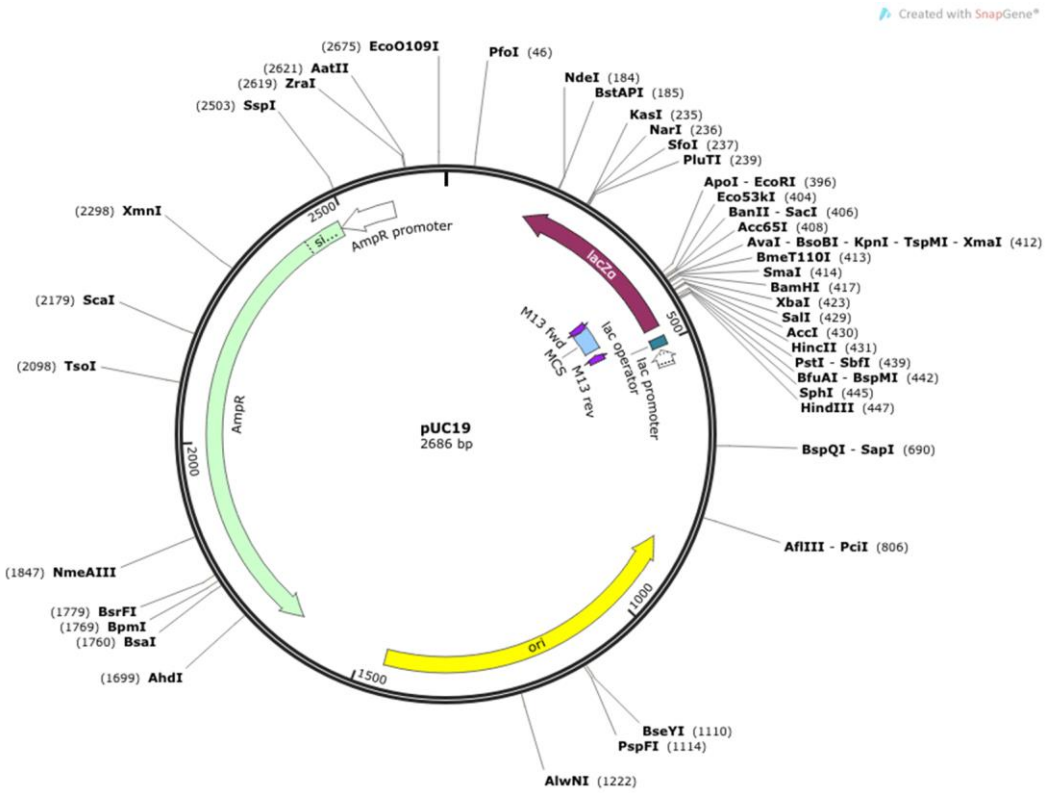

Figure App. 1. The pUC19 map used as a positive control in *E. coli* transformation http://www.snapgene.com/resources/plasmid_files/basic_cloning_vectors/pUC19/

The pUC19, a standard plasmid cloning vector in *E. coli* with 2,686 bp in length carrying ampicillin resistance gene was used as positive control in transformation to check the transformation efficiency of competent *E.coli* cells.

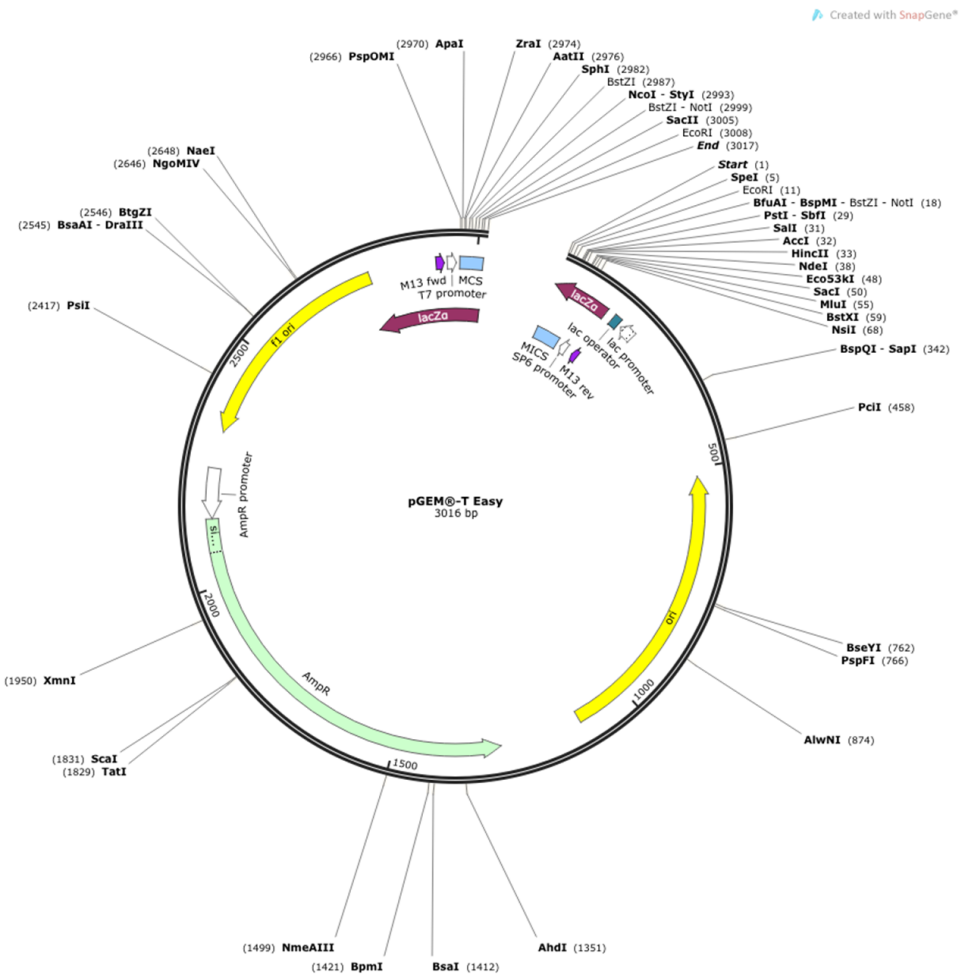

Figure App. 2. The PGEM®-T Easy vector system I map used in TA-cloning http://www.snapgene.com/resources/plasmid_files/basic_cloning_vectors/pGEM-T_Easy_(linearized)/

The pGEM®-T Easy vector system I was obtained from promega used in ligation or TA cloning. It is a linearised T-overhangs vector with 3,015 bp in length containing ampicillin resistance gene for bacterial selection and *LacZ* gene coding for β-galactosidase used as reporter gene for blue/white selection of recombinant.

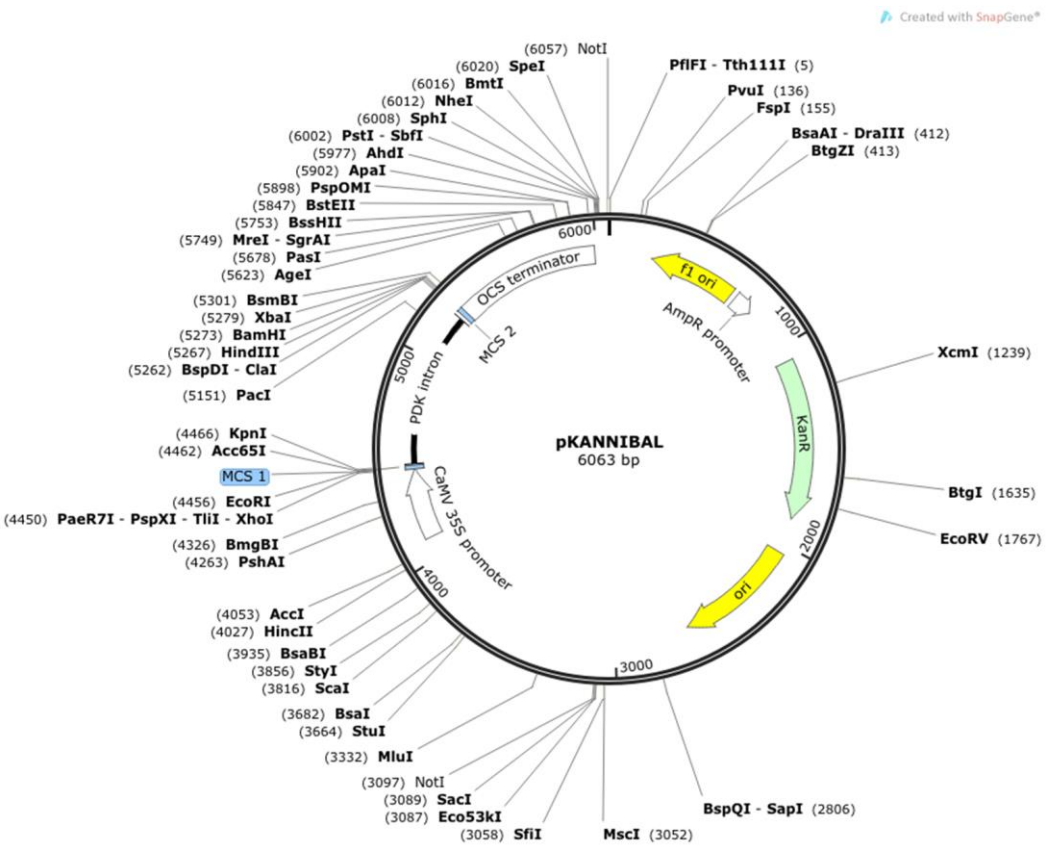

Figure App. 3. The pKANNIBAL vector for creation of hpRNA structure.
http://www.snapgene.com/resources/plasmid_files/plant_vectors/pKANNIBAL/

The pKannibal vector with 6,063 bp in length and bacterial kanamycin resistance was used to create a hairpin RNA (hpRNA) structure linked by an intron for silencing gene(s) in plants. The target gene (DNA fragment) was inserted into the vector using conventional restriction enzyme digestion and DNA ligation techniques.

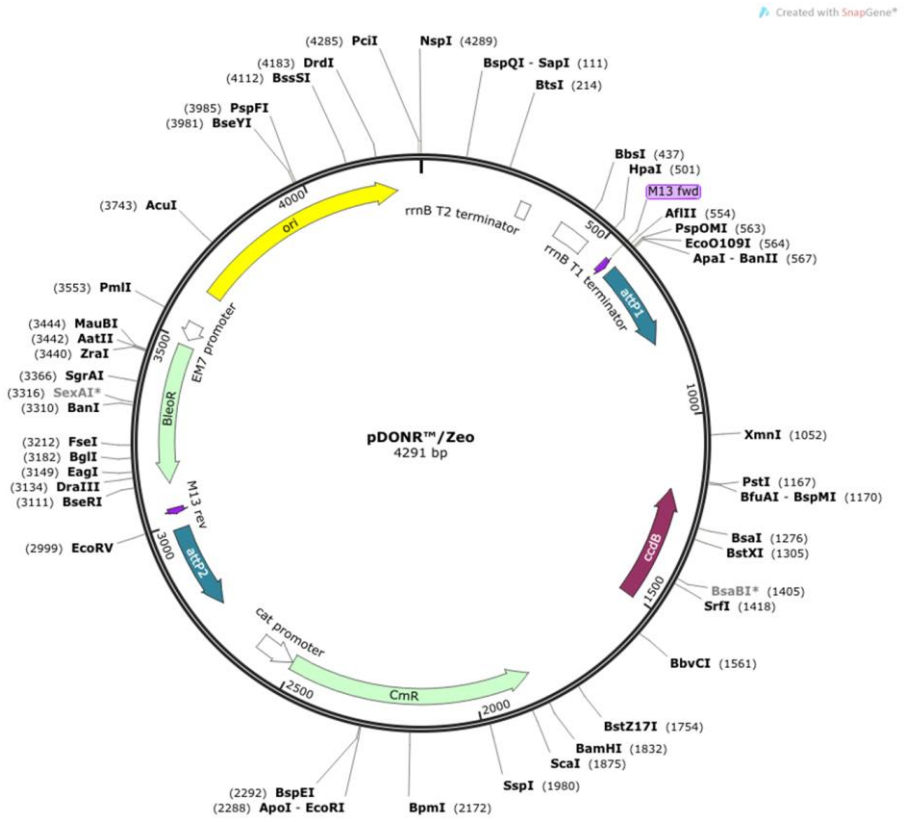

Figure App. 4. The pDONR/Zeo^{TM}vector for creation of an entry clone in the gateway cloning http://www.snapgene.com/resources/plasmid_files/gateway_cloning_vectors/pDONR_Zeo/

The pDNOR^{TM} vector used in this experiment was the one that possess Zeocin^{TM} resistance gene for selection in *E. coli* and has 4,291 bp in length. It was used in BP recombination reaction to create an entry clone.

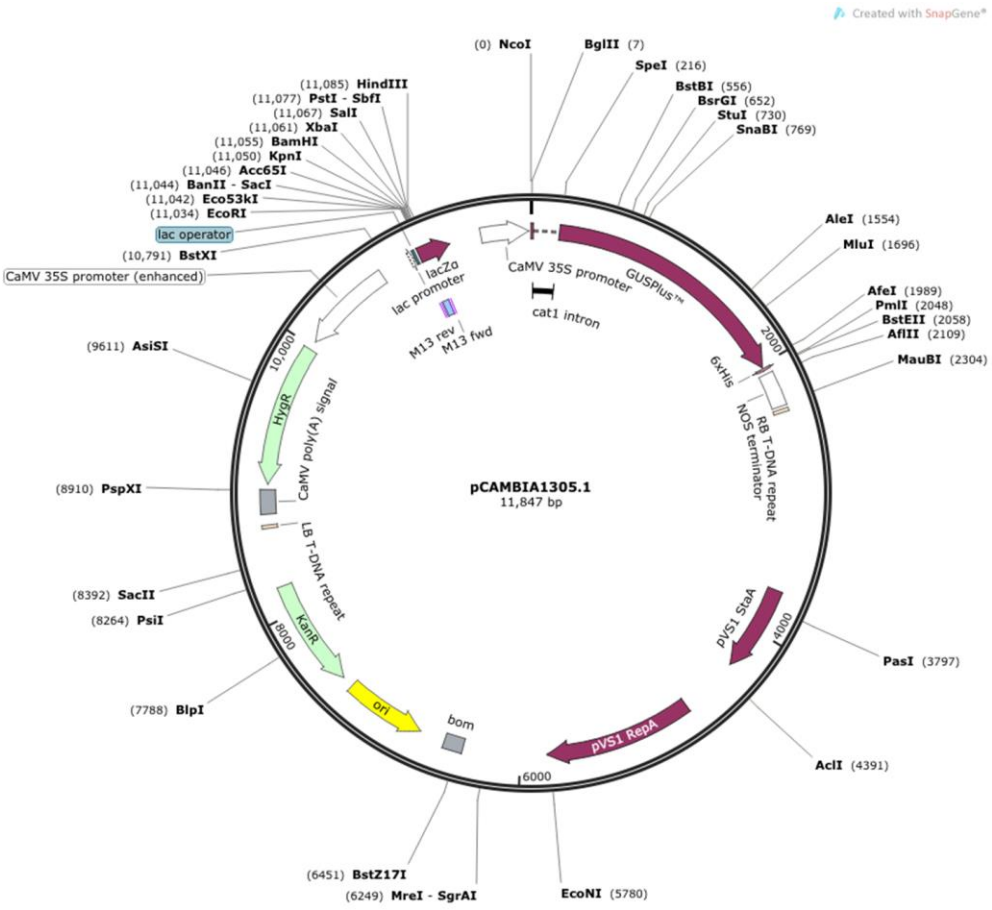

Figure App. 5. The pCAMBIA 1305.1 vector backbone used to create an expression vector

http://www.snapgene.com/resources/plasmid_files/plant_vectors/pCAMBIA1305.1/

Appendix 2. PCR set up for gene constructs creation

1. General PCR set up

Table App. 2.1. Composition of reaction mixture using Taq DNA polymerase

Component	Volume	Final concentration
10x standard Taq reaction buffer	2.5 µL	1x
10 mM dNTPs	2 µL	200 µM each
Taq DNA polymerase	0.125 µL	0.025 U/µL
10 µM forward primer	1 µL	0.4 µM (0.05-1 µM)
10 µM reverse primer	1 µL	0.4 µM (0.05-1 µM)
Template DNA	1 µL	500 ng
Nuclease-free water	to 25 µL	

Table App.2.2. PCR reaction set up using Taq DNA polymerase

Step	Temp (°C)	Time
Initial denaturation	94	2 min
Denaturation	94	30 sec
Annealing	55-60	30-60 sec
Elongation	72	60 sec/kb
Final elongation	72	10 min
Final hold	14	∞

Table App.2.3. Composition of reaction mixture using Q5 HF DNA polymerase

Component	Volume	Final concentration
5x Q5 reaction buffer	5 µL	1x
10 mM dNTPs	2 µL	200 µM each
5x HF DNA polymerase	0.25 µL	0.02 U/µL
10 µM forward primer	1.25 µL	0.5 µM (0.05-1 µM)
10 µM reverse primer	1.25 µL	0.5 µM (0.05-1 µM)
Template DNA	1 µL	500 ng

Nuclease-free water	to 25 µL

Table App.2.3. PCR reaction set up using Q5 HF DNA polymerase

Step	Temp (°C)	Time
Initial denaturation	94	2 min
Denaturation	94	30 sec
Annealing	55-60	30 sec
Elongation	72	30 sec/kb
Final elongation	72	5 min
Final hold	14	∞

2. Creation of overexpression constructs for At3g09260 (BGLU23)

Table App.2.4. Master mix composition with Q5 High-fidelity DNA polymerase

Component	**25 µL reaction**	**50 µL reaction**	**Final concentration**
10 mM dNTPs	2µL	1µL	200 µM each
5x Q5 reaction buffer	5 µL	10µL	1x
Q5 High-fidelity DNA polymerase	0.125 µL	0.25 µL	0.02 units/µL
10 µM Forward primer	1.25µL	2.5µL	0.5µM
10 µM Reverse primer	1.25µL	2.5µL	0.5µM
Template DNA	1.25µL	2.5µL	< 1,000 ng
Nuclease-free water	To 25 µL	To 50 µL	

Table App. 2.5. PCR conditions for full-length cDNA amplification

Step		**Temperature (°C)**	**Time**
Initial denaturation		94	2 min
Denaturation	30 cycles	94	30 sec
Annealing		60	1 min
Elongation		72	2 min
Final elongation		72	5 min
Final hold		14	Forever
PCR product size		1595	

PCR Template : cDNA of At3g09260 (BGLU23)

Forward primer : At3g09260_Fwd Primer3

Reverse primer : At3g09260_Rev Primer3

Table App. 2.6. Step 1 of Gateway PCR: Creating 12 *att*B PCR product

Step		**Temperature (°C)**	**Time**
Initial denaturation		94	2 min
Denaturation	10 cycles	94	30 sec
Annealing		60	1 min
Elongation		72	2 min
Final elongation		72	5 min
Final hold		14	Forever
12 *att*B PCR product size		1619 bp	

PCR Template : PCR product (full-length cDNA)
Forward primer : At3g09260_12*att*B1_FWD
Reverse primer : At3g09260_12*att*B2_REV

Table App. 2.7. Step 2 of Gateway PCR: Creating full *att*B PCR product

Step		**Temperature (°C)**	**Time**
Initial denaturation		94	2 min
Denaturation	5 cycles	94	30 sec
Annealing		45	30 sec
Elongation		72	2 min
Denaturation	20 cycles	94	30 sec
Annealing		55	30 sec
Elongation		72	2 min
Final elongation		72	5 min
Final hold		14	Forever
*att*B PCR product size		1653 bp	

PCR template : 12 *att*B PCR product

Forward primer : *att*B1 adapter_FWD

Reverse primer : *att*B2 adapter_REV

3. Construction of RNAi vector for Cassava4.1_006629 (*MeSGT*1)

3.1. Amplification of target RNAi region (sense and anti-sense orientation)

Table App. 2.8. Amplification of target RNAi region

Step		**Temperature (°C)**	**Time**
Initial denaturation		94	2 min
Denaturation	30 cycles	94	30 sec
Annealing		55	30 sec
Elongation		72	1 min
Final elongation		72	10 min
Final hold		14	Forever
PCR product size		332 bp	

PCR template : Cassava genomic DNA
Forward primer : Cassava4.1_006629B_Fwd Primer1
Reverse primer : Cassava4.1_006629B_Rev Primer1

3.2. Step 1 of Gateway PCR: Creating 12 *att*B PCR product

PCR template : PCR product (332 bp DNA fragment)

Sense orientation

Forward primer : Cassava4.1_006629S_12*att*B1_FWD

Reverse primer : Cassava4.1_006629S_KpnI_REV

Anti-sense orientation

Forward primer : Cassava4.1_006629AS_12*att*B2_FWD

Reverse primer : Cassava4.1_006629AS_ClaI_REV

Table App. 2.9. Creating 12 *att*B PCR

Step		**Temperature (°C)**	**Time**
Initial denaturation		94	2 min
Denaturation	10 cycles	94	30 sec
Annealing		55	1 min
Elongation		72	2 min
Final elongation		72	5 min
Final hold		14	Forever
12 *att*B PCR product size		356 bp	

3.3. Step 2 of Gateway PCR: Creating *att*B PCR product

PCR template : PCR product (12 *att*B PCR product)

Sense orientation

Forward primer : *att*B1 adapter primer

Reverse primer : Cassava4.1_006629S_KpnI_REV

Anti-sense orientation

Forward primer : *att*B2 adapter primer

Reverse primer : Cassava4.1_006629AS_ClaI_REV

Table App. 2.10. Creating *att*B PCR product

Step		**Temperature (°C)**	**Time**
Initial denaturation		94	2 min
Denaturation	5 cycles	94	30 sec
Annealing		55	1 min
Elongation		72	30 sec
Denaturation	20 cycles	94	30 sec
Annealing		60	1 min
Elongation		72	30 sec
Final elongation		72	5 min
Final hold		14	Forever
*att*B PCR product size		373 bp	

3.4. Creating *att*B PCR product with addition of restriction site

PCR template : PCR product (*att*B PCR product)

Sense orientation

Forward primer : Cassava4.1_006629S_XhoI-*att*B1_FWD

Reverse primer : Cassava4.1_006629S_KpnI_REV

Anti-sense orientation

Forward primer : Cassava4.1_006629AS_XbaI-*att*B2_FWD

Reverse primer : Cassava4.1_006629AS_ClaI_REV

Table 2.11. Creating *att*B PCR product with addition of restriction site

Step		**Temperature (°C)**	**Time**
Initial denaturation		94	2 min
Denaturation	5 cycles	94	30 sec
Annealing		55	1 min
Elongation		72	30 sec
Denaturation	20 cycles	94	30 sec
Annealing		60	1 min
Elongation		72	30 sec
Final elongation		72	5 min
Final hold		14	Forever
*att*B PCR product size		383 bp	

Appendix 3. Reagents for southern blotting

1. 20x SSC	**1 L**
300 mM Sodium citrate dehydrate	88.23 g
3 M NaCl	175.32 g
pH 7.6, autoclave and store at room temp. (RT)	

2. 20% SDS	**1 L**
Sodium dodecyl sulphate	200 g

3. Depurinating solution	**1L**
250 mM HCl (37%)	25 mL
H_2O	975 mL
Store at RT	

4. Denaturing solution	**1L**
500 mM NaOH	20 g
1.5 M NaCl	87.66 g

5. Neutralisation solution	**1 L**
500 mM Tris	60.57 g
1.5 M NaCl	87.66 g
1 mM EDTA	0.37 g
pH 7.2, autoclave and store at RT	

6. W1	**1 L**
2x SSC	100 mL
0.1% SDS	5 mL
Prepare fresh, store at RT	

7. W2	**1 L**
0.2x SSC	10 mL
0.1% SDS	5 mL
Prepare fresh, store at RT	

8. W3	**1 L**
0.1x SSC	5 mL
0.1% SDS	5 mL
Prepare fresh, store at RT	

9. WB	**1 L**
0.3% Tween20	3 mL
B1 solution	997 mL

10. B1 solution	**1 L**
100 mM Maleic acid	11.6 g
150 mM NaCl	8.77 g

pH 7.5 (adjusted with 7-8 g NaOH), autoclave and store at RT

11. B2	**1 L**
Blocking powder	1 g
B1 solution	100 mL

Prepare fresh

12. B3	**1 L**
100 mM Tris-HCl pH 9.5	5 mL
100 mM NaCl	5 mL
50 mM $MgCl_2$	2.5 mL

Prepare fresh